Characterisation of Chemical Processes Operating within a Biological Wastewater Treatment Plant

Thesis

Characterisation of Chemical Processes Operating within a Biological Wastewater Treatment Plant

A thesis submitted for the degree of

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to

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by

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ABSTRACT

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ABSTRACT

The activated sludge process used in biological wastewater treatment plants utilises a mixed consortia of microorganisms that break down complex organic compounds into smaller less toxic components. The complex mechanisms and pathways that exist within the biomass have not been studied to any great degree and as a consequence little is known about the specific fate pathways for many organic compounds being metabolised by the microorganisms. Studies carried out using a laboratory biological reactor using biomass taken from a large scale treatment plant have allowed a detailed determination of the various pathways for a range of rubber chemical compounds present in the wastewater from a rubber chemical manufacturing plant. In particular it has been found that concentration plays an important role in the metabolic pathway for many compounds: two different pathways exist depending on the concentration of a given substrate. Furthermore, it has been shown that the presence of other substrates can influence the pathway and metabolites generated during mineralisation of a substrate.

The compound mercaptobenzothiazole (MBT) is an example of a concentration dependent substrate. At low concentrations the MBT is broken down through initial methylation leading to the formation of 2-hydroxybenzothiazole and ultimately catechol. However, at higher concentrations the MBT is oxidised to form the disulphide 2,2′-dithiobisbenzo-thiazole. This disulphide is removed following oxidative cleavage of the sulphur-sulphur bridge producing a series of acid components including 2-benzothiazolesulphenic and sulphinic acids.

The compound *para*-hydroxybenzoic acid (PHBA) has been shown to be one of a series of substrates required for the production of enzymes that are part of an overall scheme in which a substance giving a characteristic 'red' colouration to the effluent is produced and/or controlled. It has been shown that an absence of PHBA leads to the loss of a secondary substrate-enzyme complex, which subsequently leads to formation of a substance with a highly coloured chromophore following the reintroduction of the PHBA substrate.

STATEMENT

The work embodied in this thesis was carried out by the author between October 1995 and December 2000 in the laboratories of Flexsys Rubber Chemicals Ltd, Ruabon, Wrexham, North Wales, under the supervision of Dr Nick Winter and Mr Howard Bowles.

I declare that the work presented is the result of my own investigations.

The material within this thesis has not been submitted, nor is currently being submitted, for any other degree.

Parts of the work have been presented as listed below.

Open University students open day for part-time external PhD research students,
Open University, Milton Keynes, September 1997 (oral presentation).

Royal Society of Chemistry 'Young Scientist Symposium',
Bangor University, Bangor, North Wales, July 1999 (oral presentation).

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Andrew Buck
December 2003

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ABBREVIATIONS

2ABT	2-aminobenzothiazole
2NS	naphthalene-2-sulphonate
4HMP	4-hydroxy-4-methyl-2-pentanone
AU	absorbance unit
BOC	British Oxygen Company
BOD	biological oxygen demand
BTH	benzothiazole
BTOH	2-hydroxybenzothiazole
BTSA	benzothiazole-2-sulphonic acid (see also $BTSO_3H$)
BTSOH	benzothiazole-2-sulphenic acid
$BTSO_2H$	benzothiazole-2-sulphinic acid
$BTSO_2Na$	sodium benzothiazole-2-sulphinate
$BTSO_3H$	benzothiazole-2-sulphonic acid (see also BTSA)
BTSOMOR	benzothiazolyl morpholinyl sulphoxide
CTAB	cetyltrimethylammonium bromide
DMS	dimethyl sulphide
DO	dissolved oxygen
DPO	diphenyl oxide
DPU	diphenylurea
GAC	granular activated carbon
HPLC	high performance liquid chromatography
LLE	liquid-liquid extraction
MAC	maximum admissible concentration
MBS	2-(4-morpholinothio)benzothiazole
MBT	2-mercaptobenzothiazole
MBTS	bis(2-benzothiazolyl) disulphide or 2,2′-dithiobisbenzothiazole
MeBBT	3-methylbenzothiazole-2-thione
MeBTH	2-methylbenzothiazole
MeMBT	2-methylmercaptobenzothiazole

MLSS	mixed liquor suspended solids
MS	metal sulphide
MSO_2BTH	2-methylsulphinylbenzothiazole
MSOBTH	2-methylsulphenylbenzothiazole
MSW	municipal solid waste
NMR	nuclear magnetic resonance
PHBA	*para*-hydroxybenzoic acid
PU	phenylurea
PVI	prevulcanisation inhibitor
RAS	return activated sludge
SPE	solid phase extraction
SRB	sulphate-reducing bacteria
TKN	total Kjeldahl nitrogen
TLC	thin layer chromatography
TN	total nitrogen
TOC	total organic carbon
UNEP	United Nations Environment Programme
US EPA	United States Environmental Protection Agency
WAS	waste activated sludge
WWTP	wastewater treatment plant

CONTENTS

CHAPTER 1
INTRODUCTION

1.1 Water sources and the need for purification

Ground water is that water which is below the Earth's surface. The interface between the upper region where air is present in the soil and underlying rocks and the level below which any pores, cracks etc. are filled with water is known as the 'water table'. Surface waters can be broadly split into two categories, salt and fresh water. Fresh water is found in rivers and lakes, the most significant example of salt (saline) water being seawater.

While much of the world's water is to be found in the oceans, it is also present in snow packs, glaciers, and polar icecaps. It is continually moving, both as a liquid and a vapour. The oceans of the world, inland lakes and rivers all act collectively as a giant 'chemical system' comprising the global biogeochemical cycles which control the movement and fate of materials, both natural and those produced by human activities on our planet (pollutants). Thus it forms a global sink because it acts as a huge-scale 'interceptor' for all manner of materials that originate from the various geospheres that exist.

According to Chester (1990), pollutant materials reach the aquatic system via a number of individual but interrelated stages namely:

Stage 1: (*Source*) ~ point of release into the environment.
Stage 2: (*Transport*) ~ movement of materials to the ocean reservoir.
Stage 3: (*Internal reactivity*) ~ the biogeochemical processes operating within the oceans.
Stage 4: (*Sink*) ~ the removal of materials from the ocean reservoir.

Water pollution is one of the most important environmental problems facing the modern world today. Faecal pollution of water, especially drinking water, has frequently caused water-borne diseases. In general, though, water-borne diseases have been well controlled, especially in developed countries (Bassett, 1992). Today, water-borne toxic chemicals pose a greater threat to the safety of water supplies in developed and developing countries alike. There are many sources of toxic chemicals in the environment, such as badly managed landfill sites, industrial pollution and pesticide runoff (British Medical Association, 1992).

Industrial wastewater represents an ever increasing volume of complex mixtures as point sources of water pollution. As a consequence, the treatment of industrial wastewater is of global importance, given the need to protect limited water resources. Various technologies are employed to treat the problem, including chemical oxidation (Lankford and Eckenfelder, 1990), photolysis (Kim and Anderson, 1994) and microbial degradation (Parsek *et al.*, 1995).

1.2 Sources of aqueous pollution

It has been estimated that on a global scale up to 70% of oceanic pollution originates from land-based sources (UNEP, 1990). It is necessary to distinguish between pollution and contamination. Although both terms mean the same to the average lay person, in scientific usage they have two distinctly different meanings. Contamination implies that a chemical is present in a given sample with no evidence of harm whereas pollution means the presence of a chemical that is causing harm or nuisance.

According to Edwards (1994), the effects of water pollution can be summarised as:

- aesthetic ~ visual nuisance caused, *e.g.*, litter, discoloration and smells
- temperature
- deoxygenation ~ acute or chronic toxicity causing damage to aquatic or human life
- acidity/alkalinity ~ disturbance of the pH regime
- eutrophication ~ nutrients giving rise to excessive growths of some organisms

Any material can become a pollutant in water causing one or more of the above effects, even everyday products such as milk or sugar. Any material entering the aquatic ecosystem can become a pollutant under the right conditions. Whether a high biological oxygen demand (BOD) waste discharge to natural waters causes a serious environmental problem depends almost entirely on the characteristics of the receiving system. If the input of BOD is greater than the ability of the receiving water to supply new oxygen then there will be a major problem of oxygen depletion and in extreme cases, total anoxia. If the BOD input and the new oxygen supply are of similar magnitude then some oxygen depletion may be observed. Only if the renewal of oxygen is much greater than the supply of BOD will the discharge be innocuous (at least in this respect).

Certain chemicals have been identified and are categorised according to 'The Dangerous Substances Directive' (76/464/EEC) which was adopted in 1976 (Harrison, 1996). In this directive chemicals are either placed on List 1, commonly referred to as the 'Black List', or List 2, the 'Grey List'. While a few years ago it would have been possible to regard only the 'Black List' substances as being priority pollutants, today it is difficult to identify one single list. Chemicals in the 'Grey List' can become priority pollutants in different contexts.

While the priority pollutants are important, the majority of pollution incidents in the UK occur as a consequence of gross organic pollution. Oil spills are cited as the main cause of pollution, a case in point being the wrecking of the *Torrey Canyon* oil tanker on the Scilly Isles in 1967, which at the time was carrying some 120 000 tonnes of crude oil.

Much of the material involved in pollution is of natural origin, only becoming a pollutant as a consequence of human activities. This is in addition to material generated by human activities. It is useful to distinguish between these two types of anthropogenic material.

Type I ~ naturally occurring material, for which the anthropogenic effect arises only when human activities result in its excessive release into the environment (*e.g.* by mining, smelting, waste incineration, sewage disposal, etc.).

Type II ~ non-naturally occurring material which has been manufactured in a laboratory or on an industrial plant, which is subsequently released into the environment.

Sources of polluting materials are as diverse as the chemical compounds available and these include those from the manufacture of products for modern day living. Examples are bulk organic chemicals, pharmaceutical compounds, paints, pigments, foodstuffs, organo-metallic compounds, etc.

The agricultural problem is not restricted to manufactured materials. Natural products from intensive farming methods such as silage production and storage, cattle slurry run-off, and dairy washings are but a few of the sources of natural pollutants.

As a consequence of modern man's thirst for a higher standard of living, there has occurred an explosion in the production of waste from the many manufacturing processes as well as that of natural wastage of human use and disposal. Most of the material from manufacture is sent for disposal by either landfill or incineration, while the majority of household waste is disposed of by landfilling. One of the problems associated with landfilling is that of leachate from the landfill site.

Currently landfill takes about 80% of municipal solid waste (MSW) (Department of Environment, 1998). It is therefore a critical element in a waste management strategy, since despite the best attempts at waste minimisation, recycling and recovery, its use is unavoidable. The challenge is to design and manage the process of landfilling in a sustainable manner so as not to leave a long-term potential for environmental damage.

1.3 Sewage

Sewage is broadly split into two types, domestic and trade (industrial) depending on the origin. Sewage sludge is the product of initial physical treatment of raw sewage, treatment processes include screening to remove large objects followed by grit removal. After screening and grit removal the raw sewage is then subjected to primary and secondary sedimentation. The products of these processes are disposed of either by landfill or incineration: the screening products and grit are generally fit for landfilling whereas the products of primary and secondary sedimentation are in some cases treated further via anaerobic digestion and following dewatering sent for landfill and/or incineration.

Domestic sewage, with or without the presence of industrial wastes, probably represents the commonest and most widespread contaminant of inshore and near shore waters. Over 10^6 tonnes of dry sewage sludge are produced in the UK alone each year (Department of Environment, 1995). At present a significant amount of both untreated and treated sewage and sewage sludge reaches coastal waters. For example, over 5×10^6 wet tonnes per annum (equivalent to approximately 5×10^5 dry tonnes per annum) of sewage sludge was dumped in the North Sea by the UK in 1990 (Oslo and Paris Commissions, 1993).

Sewage poses aesthetic and health risks to human populations and also acts as a medium whereby a wide variety of contaminants reach the marine ecosystem. Sewage may reach the seas in a number of different forms, ranging from untreated raw sewage discharges, through various degrees of treated discharge, to the dumping of associated sewage sludge at marine sites.

In 1991, the countries within the European Union, having a domestic population of 345 million, operated 40 300 sewage treatment works, which generated some 6.5 million tonnes of dry solids per year (Hall and Dalimier, 1994). This was an increase of 18% from the 5.5 million tonnes of dry sludge produced in 1987 (Hall, 1992). In the USA, over 7 million tonnes of dry sewage sludge were produced in 1990 (McGhee, 1991). In Japan, there has been a striking change in the provision of sanitary services during the last 30 years. In the early 1950s, almost all the population used a night soil collection system with relatively few sewage treatment plants being available. Since 1955, the urban areas have shown a rapid growth in the installation of flush toilets and sewer connections through a series of five-year programmes (Tebbutt, 1992).

1.3.1 Disposal of sewage sludge

Sludge can be broadly split into two categories: (a) municipal sewage sludge and (b) industrial sewage sludge. The disposal routes for each will depend on the sludge composition.

Residues of treatment of urban wastewater can be applied to the land to serve as a fertiliser and soil conditioner. Sludge recycling as fertiliser has a number of advantages, one being the return of organic material into the biogeochemical cycle. It also serves to reduce the need for artificial fertilisers, the manufacture of which is energy expensive. The application of sewage sludge as land fertiliser in 1990 took 43% of the total in the UK and 22% of the total in the USA (Harrison, 1996).

By contrast, industrial sludge containing a high organic chemical load would normally be disposed of by either dumping at sea (no longer a current practice however), incineration, or landfill at a controlled site. In Europe, only three countries practised dumping at sea,

Ireland 35% of the sludge produced, the UK 30%, and Spain 10%. Disposal at sea was controlled in accordance with certain requirements *e.g.*, the Oslo Convention for the protection of the North Sea and North East Atlantic, and in accordance with licences issued under national legislation which take into account the quantity as well as the nature of the receiving area (Matthews, 1992). Disposal of sewage sludge at sea ceased in 1998, when the North Sea Conference Agreement came into force.

1.4 Reasons for sewage treatment

Fresh water is indisputably an essential resource, several litres (~2.5 – 4.0) must be consumed daily if an individual is to stay alive. A minimum estimate for total personal use (cooking, bathing, flushing away waste) is fifty litres per day (Gleick, 1996). Additionally water is essential for crop production. Irrigation with wastewater is both disposal and utilisation and indeed is an effective form of wastewater disposal (as in slow-rate land treatment). However, some degree of treatment must normally be provided to raw municipal wastewater before it can be used for agricultural or landscape irrigation or for aquaculture. The quality of treated effluent used in agriculture has a great influence on the operation and performance of the water-soil-plant or aquaculture system. In the case of irrigation, the required quality of effluent will depend on the crop or crops to be irrigated, the soil conditions and the system of effluent distribution adopted.

The current and projected future rates of water consumption are not sustainable with present technologies: it was estimated in 1998 that 1.5 – 2.0 billion people were falling short of the daily requirement for personal use. On a global scale, humanity is consuming approximately fifty percent of the accessible fresh water for personal, industrial and agricultural uses (Postel *et al.*, 1996).

Developments in the technology of agricultural use of water could enhance the efficient use of water for crop production and reduce the per capita requirement. Only around 60% of water diverted to fields for irrigation is utilised by crops, while 40% is lost between the irrigation water source and the crops (Zilberman, 1997). According to the World Resources Institute (1997) only 25% of all water diverted to irrigation systems gets to crop plants, due

to losses from leaking pipes, evaporation in reservoirs and conveyance channels, and from the use of inefficient irrigation systems. More fresh water could be created by the treatment of wastewater. However, reuse of wastewater is only feasible for the portion of that water supplied as non-consumption water. Since agriculture is by far the largest consumer of water, and its use is consumptive, irrigation water that has been evapotranspired can not be reused. Rapid growth in the demand for high quality water coupled with a natural shortage and continuous restrictions in supply, primarily in arid and semi-arid regions, have accelerated the search for alternative sources. The additional sources include fresh high quality run-off water, brackish water and treated sewage. In regions with limited natural water sources, treated wastewater, primarily urban sewage, can be utilised for agriculture, industry, recreation and recharge of aquifers (Bouwer, 1989; Asano and Mills, 1990; Asano *et al.,* 1992). Most importantly, effluent application for irrigation simultaneously solves water shortage and wastewater disposal problems.

1.5 Wastewater treatment processes

An understanding of the sources of water for communities must be taken into consideration when devising water quality standards. Modern analytical techniques today are capable of much lower detection limits compared to levels say 10 years ago. In the past it has been the practice to set standards on the then-perceived practical quantitation limit. Standards set in this way are difficult to defend logically and are very quickly outdated by improvements in analytical methodology and available instrumentation. A standard which is desirable and relatively easily achievable for one water source may be less so when applied to a more challenging source.

1.5.1 Wastewater treatment

There are three main types of water treatment processes commonly applied, namely physical processes, biological processes and chemical processes. However, there is no specific order in which they may be applied. In general, physical processes precede biological processes; chemical treatment may be applied either before, or may even replace, biological processes.

Each type of treatment process provides a valuable part of the whole treatment stream, but each has deficiencies and may fail to remove a key pollutant or conversely introduce new components into the treated water. No treatment removes 100% of a particular component but may operate at the 99% and 99.9% level. The impact of an order of magnitude difference in a standard could be very considerable in terms of a plant's ability to deal with peak loading and achieve a compliant product. Most treatment works rely on a series of 'barriers' to pollution and include more than one of the above processes. The tighter the standard the higher the cost of meeting that standard.

1.5.2 Physical treatment

By the 18th century, filtration of particles was established as an effective way of clarifying cloudy water. Physical separation techniques rely on the physical properties of the different impurities present, such as specific gravity, viscosity or particle size. These processes involve adsorption, sedimentation, flotation and physical filtration. Physical treatment *per se* does not change the nature of the constituents in the water, but it removes particulate matter, which might support both microbial and chemical pollutants. Latterly, membrane filtration has become more widely used to remove pathogens and undesirable chemical species by using pore sizes at the micrometre or nanometre level and will continue as a viable process both alone and as a precursor for other processes.

1.5.3 Biological treatment

Biological treatment processes rely on the development of natural biological ecosystems within treatment units to adsorb and biodegrade both soluble and colloidal components of the water being treated. Although these systems may be either aerobic or anaerobic, it is usual to adopt aerobic systems in drinking water treatment. Typical aerobic biological systems include slow sand filters, granular activated carbon (GAC) and activated sludge systems.

1.5.4 Chemical systems

In a bid to increase the efficiency of filtration and disinfection, and to provide treatment for specific contaminants, chemical-based systems were developed. Some are based on

chemical coagulation, flocculation and settlement or flotation, others use strong oxidants to cleave larger molecules followed by adsorption on GAC. These systems are very effective in producing safe drinking water but they also suffer from disadvantages. It is a corollary of any new chemical purification system that they need further standards to control them, because the treatment chemicals themselves may leave residues or create by-products. Ozone is a classic case of a powerful treatment developed to meet a specific need, a surrogate zero standard for all pesticides, which then raised concern over the formation of bromate from bromide in raw waters. The magnitude of any proposed bromate standard may ultimately restrict the viability of the ozone treatment, thus negating the obvious benefits that are derived from its use.

Aluminium sulphate and other aluminium compounds are widely used as coagulants in wastewater treatment. Hydrolysis of aluminium sulphate causes insoluble 'flocs' to form which engulf colloidal and suspended materials in the raw water. The resultant floc can be removed by a number of physical processes, the most common technique applied being simple sand filtration, which may or may not be preceded by a sedimentation step.

Over the past decade, concerns about aluminium usage and its possible link to Alzheimer's disease has grown considerably. Empirical data exist that seem to support such a relationship. The most notable example is that reported by Martyn *et al.* (1997), who demonstrated that the frequency of Alzheimer's disease increased by 50% when the average level of aluminium in the drinking water supply reached 0.11 mg/l. It is interesting to note that the maximum admissible concentration (MAC) which is accepted in the European Community for aluminium in public drinking water is 0.20 mg/l.

During July 1988 a large quantity of aluminium sulphate was accidentally released in the reservoir at Lowermoor Treatment Works, which provided drinking water for the town of Camelford in Cornwall, England. This treatment works supplied more than 7000 properties and approximately 20 000 local consumers and tourists in North Cornwall. The levels of aluminium increased to over 10 mg/l, well above the 0.2 mg/l limit set by the EC on palatability grounds. Despite the repeated assurances that aluminium in drinking water

posed no health hazards, many people did in fact suffer from persistent medical problems (Lowermoor Incident Health Advisory Group, 1991).

1.6 Physical processes

At the present time, most of the processes used for wastewater treatment are undergoing continual and intensive investigation from the standpoint of implementation and application. As a result, many modifications and new operations and processes have been developed and implemented; more need to be made to meet the increasingly stringent requirements for environmental enhancement of watercourses.

1.6.1 Screening

The first unit operation encountered in wastewater treatment plants is screening. A screen is a device with openings, generally of uniform size that is used to retain the coarse solids found in the wastewater. The screening element may consist of parallel bars, rods or wires, grating, wire mesh or perforated plate and the openings may be of any shape but generally are circular or rectangular slots. A screen composed of parallel bars or rods is called a 'rack'. Although a rack is a screening device, the term 'screen' should be limited to the type with wire or cloth or perforated plates. However, the function performed by a rack is called screening, and the materials removed by it are known as screenings or rackings.

According to the methods used to clean them, racks and screens are designated as hand cleaned or mechanically cleaned. Typically, racks have clear openings (spaces between bars) of 25 mm or more. Screens have openings of 6 mm or less. In wastewater treatment, bar racks are used to protect pumps, valves, pipelines etc., from damage or clogging by rags and other large objects. Industrial waste plants may or may not need them, depending on the character of the incoming wastes.

In the 1920s and earlier, fine screens of inclined disk or drum type, whose screening media consisted of bronze or copper plates with milled slots, were installed in place of sedimentation tanks for primary treatment. Since the early 1970s, there has been a resurgence of interest in the field of wastewater treatment in the use of screens of all types. The applications range from primary treatment to the removal of the residual suspended

solids from biological treatment processes. To a large extent, this renewed interest developed because better screening materials and hence better screening devices are now available.

1.6.2 Sand filters

The slow sand filters were first introduced for the treatment of London's water supply in 1829 (Holden, 1970) and they have remained popular ever since; indeed some companies are still installing them and others are modifying them to include a GAC layer (Bauer *et al.*, 1995). The basis for the treatment system is concentrated biological activity, which is also fundamental to the safety of many natural groundwaters. Slow filters rely on a biological surface film on and into which contaminants in the feed water are adsorbed and biodegraded. Accordingly sand filters are simultaneously physical and biological in their action.

The advantages of such a plant are that (a) it does not introduce a chemical by-product into the treated water, and (b) as well as removing biodegradable pollutants it provides an effective barrier to pathogenic organisms. The disadvantage is that it is not able to remove complex natural or synthetic organic compounds such as humic acids, geosmin or pesticides. Therefore, alone it is not suitable for treating complex river waters where a more robust treatment is necessary. Biological systems are also prone to being poisoned or damaged by components in the feed water, which may be toxic to the microorganisms involved (see below).

1.6.3 Sedimentation

Sedimentation is the separation from water by gravitational settling of suspended particles that are denser than water. It is one of the most widely used unit operations in wastewater treatment. The terms sedimentation and settling are used interchangeably. A sedimentation basin may also be referred to as a sedimentation tank, settling basin or settling tank.

Sedimentation is used for grit removal, particulate matter removal in the primary settling tank, biological floc removal in the activated sludge settling tank and chemical floc removal when the chemical coagulation process is used. It is also used for solids

concentration in sludge thickeners. In most cases, the primary purpose is to produce a clarified effluent and/or feed to a biological treatment plant.

When a liquid containing solids in suspension is placed in a relatively quiescent state, those solids having a higher specific gravity than the liquid will tend to settle, and those with a lower specific gravity will tend to rise. These principles are used in the design of sedimentation tanks for treatment of wastewaters. The objective of treatment by sedimentation is to remove readily settleable solids and floating material and thus to reduce the suspended solids content.

Primary sedimentation tanks may provide the principal method of wastewater treatment, or they may be used as a preliminary step in a multiple process. When these tanks are used as the only means of treatment, they provide for the removal of settleable solids capable of forming sludge banks in the receiving waters and free oil and grease and other floating material. When they are used as a primary step to biological treatment their function is to reduce the load on the biological treatment units.

1.7 Chemical unit processes

Those processes used for the treatment of wastewater in which change is brought about by means of or through chemical reaction are known as chemical unit processes. In the field of wastewater treatment, chemical unit processes usually are used in conjunction with the physical and biological unit processes to meet treatment objectives. In considering the application of the chemical unit processes, it is important to remember that one of the inherent disadvantages associated with the use of chemical unit processes, as compared with the physical unit operations, is that they are additive processes. In most cases, something is added to the wastewater to achieve the removal of something else. As a result, there is usually a net increase in the dissolved constituents in the wastewater.

For example, where chemicals are added to enhance the removal efficiency of plain sedimentation, the total dissolved solids concentration of the wastewater is always increased. If the treated wastewater is to be reused, this can be a significant factor. This additive aspect is in contrast to the physical unit operations and the biological unit

processes, which may be described as being subtractive, in that material is removed from the wastewater.

1.7.1 Chemical precipitation

Chemical precipitation in wastewater treatment involves the addition of chemicals to alter the physical state of dissolved and suspended solids and facilitate their removal by sedimentation. In some cases the alteration is slight and removal is effected by entrapment within a voluminous precipitate consisting primarily of the coagulant itself. Another result of chemical precipitation is a net increase in the dissolved constituents in the wastewater.

Over the years a number of different substances have been used as precipitants. The most common are listed in Table 1.1.

Table 1.1 Common precipitants.

Chemical	Formula	Molecular Weight
alum	$Al_2(SO_4)_3.18H_2O$ [a]	666.7
ferrous sulphate	$FeSO_4.7H_2O$	278
lime	$Ca(OH)_2$	56 (as CaO)
ferric chloride	$FeCl_3$	162.1
ferric sulphate	$Fe_2(SO_4)_3$	400

[a]Number of bound water molecules can vary from 12 to 18.

1.7.2 Flocculation

Many industrial processes such as papermaking, mineral processing, water treatment and sludge dewatering involve solid-liquid separation using polymeric flocculants. In many of these applications, use of combinations of oppositely charged polyelectrolytes under suitable conditions enhances the flocculation (Yu and Somasundaran, 1996). Such combinations are increasingly used as flocculants in the industry. A limited amount of mechanistic or molecular level research has been done in the past. As a result, the fundamentals of this complex process, for example the influence of polymer molecular weight and polymer conformation on the flocculation process, are far from being well understood.

It has been known for some time that a pair of oppositely charged polymers can produce synergism in the flocculation of paper pulp. Petzold *et al.* (1996) studied the effect of charge ratios of the two oppositely charged polymers and the influence of polymer molecular weight on flocculation. The conclusions of this work were that the strongest flocculation and best retention were obtained when highly charged polycations were used in conjunction with a very high molecular weight polyanion (polyacrylamide type).

Bioflocculation is salient in determining the success of an activated sludge process and the subsequent treatment of surplus activated sludge. This is because the characteristics of activated sludge flocs influence a number of important processes such as substrate utilisation, settling and thickening as well as dewatering properties. The presence of large settleable floc structures results in a sludge with favourable settling and thickening properties which helps to reduce solids carryover in the final effluent.

It appears from the literature that the size and morphology of activated sludge flocs are dependent on the operating conditions employed in an activated sludge plant. Karr and Keinath (1978), Eriksson and Hardin (1984), and Knocke and Zentkovich (1986) all examined the influence of sludge age on the size distribution of activated sludge flocs, and found that size increased with the operational sludge age. Moreover, flocs from plants operated with long sludge ages had large compact structures with short supporting filaments. In contrast the activated sludge flocs from plants operated with short sludge ages were small diffuse structures with poor settling characteristics. The findings of Pitman (1975) were similar, and the author also investigated the effects of plant operation on effluent quality.

1.8 Biological processes

The objectives of the biological treatment of water are to coagulate dissolved organics and remove the non-settleable colloidal solids and to stabilise the organic matter. For domestic wastewater, the major objective is to reduce organic content and in many cases the nutrients, such as nitrogen and phosphorus. For agricultural return wastewater, the objective is to reduce the nutrients specifically nitrogen and phosphorus that are capable of

stimulating the growth of aquatic plants. For industrial wastewater, the objective is to remove or reduce the concentration of organic and inorganic compounds. Because many of these compounds are toxic to microorganisms, some form of pre-treatment may be required.

1.8.1 Bacteria

The bacteria and the blue-green bacteria formerly known as blue-green algae, together constitute the known *Prokaryotae* of all organisms. This group is of the most significance to the water treatment industry, since biological wastewater treatment processes rely almost exclusively on the activity of bacteria. Most prokaryotes are unicellular organisms with a single chromosome which is unbounded by a nuclear membrane. No internal organelles or specialist structures, such as are found in the more complex eukaryotic cells, are evident. Although some prokaryotic cells display a certain degree of structural differentiation their major diversity lies in their metabolic characteristics, *i.e.*, their food sources and methods of producing energy.

These characteristics, which are defined in terms of carbon source, energy source and the electron donor utilised, are indicative of the enzymatic composition of different microorganisms and can thus be used to distinguish specific nutritional categories. Bacteria that obtain carbon from an inorganic source (*i.e.*, carbon dioxide, CO_2) are termed autotrophs, while those that utilise organic carbon for cellular synthesis are called heterotrophs. Similarly two sources of energy can be identified: phototrophic microorganisms use a light source to produce energy from the reaction with photosynthetic pigments such as chlorophyll, while chemotrophic microorganisms oxidise organic or inorganic chemical compounds. The electron donor involved in such reactions may be organic or inorganic, giving a further division of organotrophism and lithotrophism respectively. The above categorisations are frequently combined to give a full description of a bacterium's metabolic requirements. Thus a phototrophic organism with an inorganic electron donor such as sulphide would be a photolithotroph, whereas one with an organic electron donor such as acetate would be a photoorganotroph. Likewise, chemotrophic organisms obtaining energy from oxidation of an inorganic electron donor such as

hydrogen are chemolithotrophs and those oxidising reduced organic compounds such glucose are chemoorganotrophs.

1.8.2 Aerobic wastewater treatment processes

The development of wastewater treatment processes was originally prompted by the frequent outbreaks of waterborne diseases in densely populated areas, and to a lesser extent, the need to reduce the odours associated with putrefaction. Although removal of pathogens remains an important aspect of treatment, the prevention of organic, and latterly nitrogen and phosphorus, pollution of surface waters has become a major objective.

Aeration was initially employed as a possible means of reducing odours in wastewater holding tanks. It was also noted that during soil percolation, if the flow of wastewater was stopped intermittently to allow aeration of the soil, improved treatment efficiency and loading capacity was obtained. It was soon realised that purification was due to biochemical oxidation and a major step forward was the development of a system that incorporated a continuous inflow of wastewater and continuous aeration. Such a system was the forerunner of the activated sludge process, while the development of soil percolation systems led to the process known as trickling or percolating filtration. These two processes, or modifications thereof, are by far the most frequently used in the UK for the treatment of both domestic and industrial wastewater today.

Wastewater treatment plants reduce the pollution of industrial and municipal waters. The effectiveness of their operation is an important aspect in order to reduce levels of contamination of outflowing waters. Current systems depend on various combinations of chemical, biological and mechanical operations for the treatment of wastewater. Activated sludge processes are very commonly used to obtain both efficient and economical wastewater systems. The success of the plants depends mainly on the expertise of both the designers and the operators of the plant (Metcalf & Eddy Inc., 1991).

1.8.3 Aerobic pond systems

Historically, aerobic wastewater stabilisation pond systems have been a principal biological treatment method for a variety of wastewaters ranging from residential domestic

to complex industrial. They may be used alone or in combination with other treatment processes.

The term 'pond' is a somewhat imprecise term, but in general implies a biological treatment system of a lesser complexity from an engineering standpoint (but not biologically) than the more technologically advanced activated sludge and percolating filter processes. The greater the simplicity of pond designs means that they are cheaper to construct and require less intensive maintenance and control. These important advantages are, however, obtained at the expense of long retention times and a correspondingly large land area. Thus, stabilisation ponds are most frequently employed in areas where financial resources and skilled labour are at a premium, but land is not.

A pond consists of a large shallow basin normally of artificial construction, but sometimes consisting of a natural body of water, which is designed for low cost oxidation of organic wastewaters. Three major types of pond are in existence. These are described as anaerobic, facultative, and maturation. In many cases, the pond achieves the entire treatment process within one reactor.

1.8.4 Anaerobic ponds

Anaerobic ponds normally have a depth between two metres and five metres and function as open septic tanks with gas release to the atmosphere. The biochemical reactions that take place in anaerobic ponds are the same as those occurring in anaerobic digesters, with a first phase acidogenesis and a second slower phase of methanogenesis. The effluent from anaerobic ponds will require some form of aerobic treatment before discharge or use and facultative ponds will often be more appropriate than conventional forms of secondary biological treatment for application in developing countries.

1.8.5 Facultative ponds

A common type of waste stabilisation pond is known as a facultative pond, also often called an oxidation pond. Maximum biological oxygen demand (BOD) loading for this type of pond is 300 mg/l. These type of ponds are characterised by an aerobic surface zone with a gradient to an anaerobic bottom zone.

Solids in the influent to a facultative pond (see Figure 1.1) and excess biomass produced in the pond will settle out forming a sludge layer at the bottom. The sludge blanket will be anaerobic and as a result of anaerobic breakdown of organics, will release soluble organic products to the water column above.

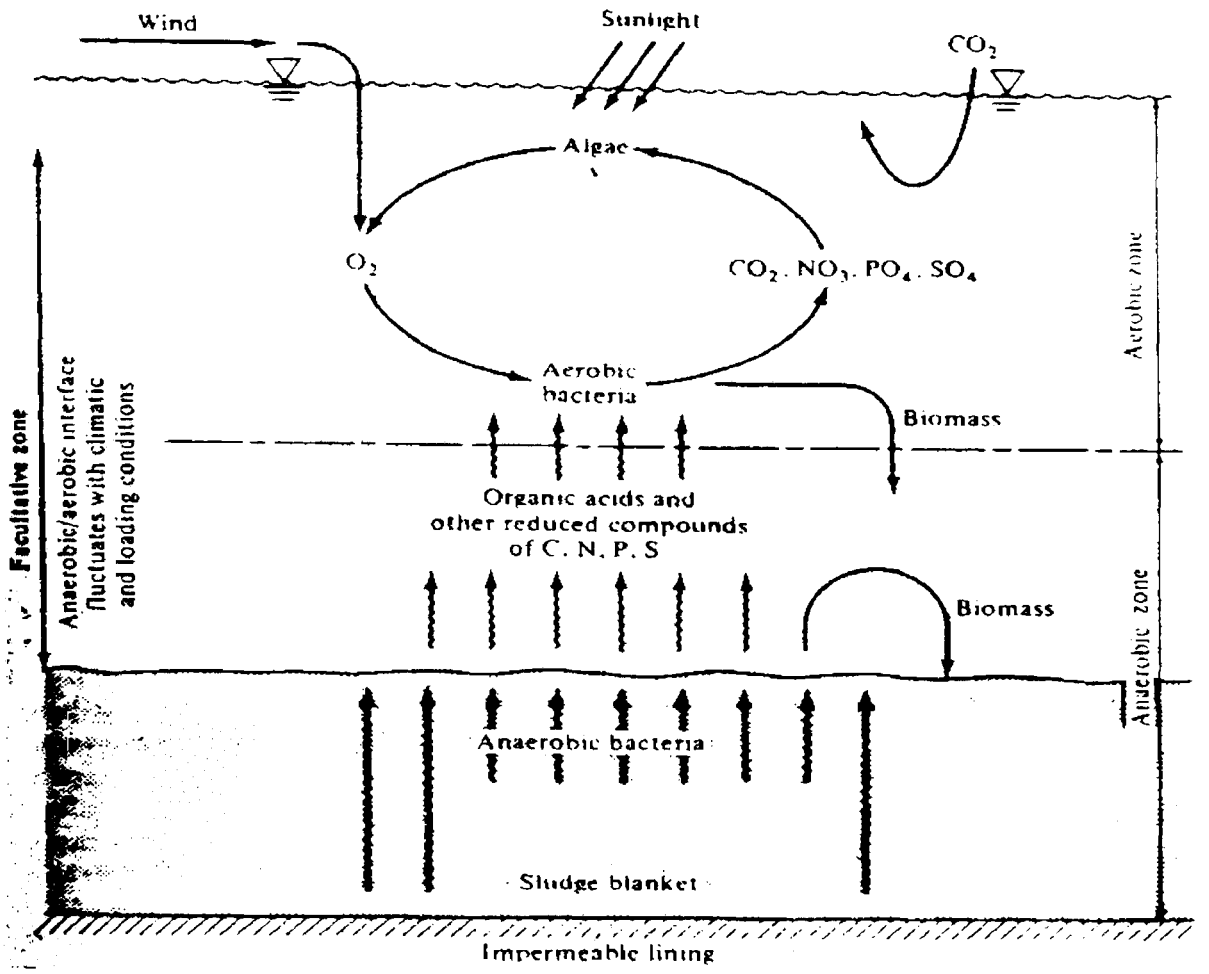


Figure 1.1 Generalised diagram of a facultative pond showing the reactions involved (Metcalf & Eddy Inc., 1991).

Organic material dissolved or suspended in the water column will be metabolised by heterotrophic bacteria, with the uptake of oxygen, as in conventional aerobic biological wastewater treatment processes. However, unlike in conventional processes, the dissolved oxygen utilised by bacteria in facultative ponds is replaced through photosynthetic oxygen production by microalgae, rather than by aeration equipment.

These type of ponds are found in hot climates where the high ambient temperature and long periods of strong sunlight create conditions that encourage algae to consume the carbon dioxide (CO_2) released by bacteria in breaking down the organic components contained in the wastewater and the uptake of nutrients (mainly nitrogen and phosphorus).

No mixing or mechanical aeration is employed, mixing of the contents being dependent on wind action and thermal gradients within the pond. The relatively quiescent conditions permit the suspended solids to settle out to the anaerobic layer at the bottom of the pond, from where desludging is done on a very infrequent basis.

1.8.6 Maturation ponds

Tertiary lagoons or maturation ponds are up to five metres deep and receive discharges from anaerobic and facultative ponds. These ponds exploit the physical and biochemical interactions that occur naturally in aquatic systems to remove pathogens, biochemical oxygen demand (BOD), ammonia, nitrates, phosphates and suspended solids. There are many thousands of systems in use all over the world. In North America alone, the US EPA (1983) reported that there were around 7 000 lagoons in use. The low operation and maintenance costs of tertiary lagoons coupled with the effective percentage pathogen removal reported in the literature (Mara and Pearson, 1987; Pearson *et al.,* 1987a; Pearson *et al.*, 1987b; Mara *et al.*, 1992a; Mara *et al.*, 1992b) have made them a popular choice for wastewater treatment.

1.8.7 Aerobic activated sludge

Many organic environmental contaminants are biodegradable under aerobic and/or anaerobic conditions. Biodegradation of these contaminants is a complex process which may be inhibited by the toxicity of the contaminant or other substances present, contaminant or nutrient nitrogen (N) and phosphorus (P) bioavailability, physical conditions (*e.g.*, temperature, salinity, pH) or microbial competition.

Biological reactors have been utilised with success for wastewater treatment for many years (Alleman and Prakasam, 1983; Peters and Alleman, 1983). Bioreactor processes can be identified by the biomass retention mechanism. Biomass grows on a carrier in attached growth *i.e.* biofilter systems or as a suspension in sludge processes. Early bioreactor tests were done by Alexander Mueller in 1865 that resulted later in the trickling filter design, which was based on attached growth on a support material in trickling filters (Corbet, 1903).

The first suspended bioreactor was the activated sludge process introduced by Arden and Lockett (1914). The next steps in bioreactor development were completely mixed stirred tank reactors in the late 1950s, fluidised-bed reactors in the late 1970s (Jerris *et al.*, 1974) and the upflow anaerobic sludge blanket reactor in 1978 (Lettinga *et al.*, 1980).

Bioreactors can be operated under aerobic or anaerobic conditions. Anaerobic bioreactors have been used since the 1980s to treat wastewater with high organic solid concentrations (Admussa and Korus, 1996). The continuous flow system can be plug-flow or completely mixed.

The basic design of an activated sludge process includes an aeration basin and a mechanical clarifier to separate the sludge from the effluent for recycle. Aeration of the water can be done by various types of air blower and aeration nozzles or injection of pure oxygen as in the Vitox™ process developed by the British Oxygen Company Ltd (BOC) (see Figure 1.2).

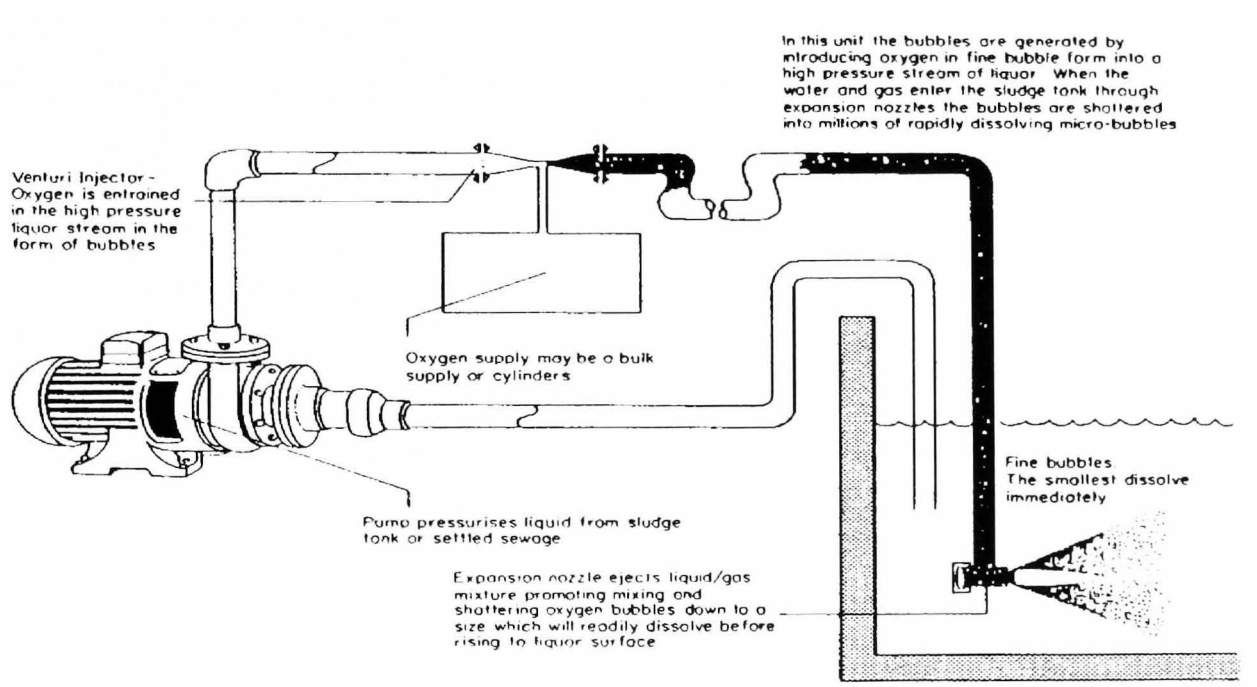


Figure 1.2 Schematic diagram of the Vitox™ high-pressure side-stream dissolver. (Diagram courtesy of BOC Ltd.).

The activated sludge process is one of the most popular and cost-effective wastewater treatment processes. The system is of considerable complexity in terms of its biological and physical aspects. Compared to many other industrial processes, the activated sludge system suffers from more frequent and severe environmental variants such as hydraulic

and organic shock loads (Zitomer and Speece, 1993). Consequently, it presents difficulties to operate and control (Marsili-Libelli, 1989).

There are numerous problems associated with the operation of any activated sludge treatment plant and these vary from site to site and process to process. It is true to say that when applied to the treatment of industrial waste the problems and complexity increase. It is not only influenced by the quality of the incoming waste stream but also the engineering of the plant, the skills and knowledge of the process manager and in particular the operator, which can all affect the final plant performance.

Aerobic digestion is typically used in smaller water treatment plants. In contrast to the anaerobic process there is no recovery of energy and the process tends to be expensive as a consequence of the energy costs of continuous aeration. However, where a plant is designed to treat flows of *ca* 20 000 cubic metres per day, aerobic activated sludge digestion appears to be the best environmental option for sludge stabilisation, incurring lower capital costs and simpler operational control requirements (Water Environment Federation, 1992). It is more flexible in operation, less prone to process failure and has a low odour potential compared to anaerobic sludge plants.

1.8.8 Trickling filters

The principle of a trickling filter is to support attached growth of bacteria by allowing the contaminated water to trickle through the support material (inert packing) due to gravity. Trickling filters are aerated from the bottom mostly by natural draught, but in some cases with an air blower. Bench scale tests with a trickling filter were carried out by van der Hoek *et al.* (1989) to remove polycyclic aromatic hydrocarbons. The test demonstrated that trickling filters are less effective than upflow fixed film reactors.

1.9 Anaerobic wastewater treatment processes

Anaerobic treatment of wastewater can be traced from the beginnings of wastewater treatment itself. Anaerobic processes have been used for the treatment of concentrated domestic and industrial wastewater for well over a century (McCarty, 1981; McCarty and Smith, 1986). The simplest, oldest, most widely used process is the septic tank (Jewell,

1987). According to Buswell (1957), a tank designed to retain solids by means of sedimentation, similar to the septic tank, was first reported in 1857.

The steep increase in energy prices in the 1970s reduced the attractiveness of aerobic methods, contributing to redirecting research efforts towards energy-saving alternatives like anaerobic treatment (van Haandel and Lettinger, 1994). High bacterial sensitivity to some environmental conditions (mainly pH, temperature and toxic compounds), long starting processes, and the production of malodorous compounds have been commonly cited as disadvantages of anaerobic treatment (Jewell, 1987).

In anaerobic processes microorganisms convert organic compounds to methane (CH_4), carbon dioxide (CO_2), cellular materials and other organic compounds. Anaerobic treatment has been used for high strength organic waste because of its potential for producing energy and a lower sludge growth rate.

Anaerobic decomposition is a three stage process:

1 hydrolysis of suspended organic solids into soluble organic compounds;

2 acetogenesis, or conversion of soluble organics to volatile fatty acids (mostly acetic acid);

3 methanogenesis, or the conversion of the volatile fatty acids into methane.

High molecular weight compounds are converted into intermediate volatile fatty acids, mainly acetic and propionic acids, by acidogenic bacteria. Acetic acid, the major intermediate in methanogenesis, is formed through the degradation of propionic and butyric acids, which involves the oxidation of hydrogen, a process involving distinct acetogenic populations. Methane can only be formed by specific methanogenic bacteria which utilise acetic acid or hydrogen. The methanogenic process relies on a balanced symbiotic relationship between interacting but metabolically distinct microbial populations of acidogenic bacteria and methanogenic bacteria (DeLorme and Kapuscinski, 1990).

1.9.1 Fluidised-bed reactor

In fluidised-bed reactors the biomass grows on granular support material. The main principle of the reactor type is the fluidisation of the bed by high recirculation rates of the

water to be treated. Recirculation of the water dilutes the concentration of the influent to a non-toxic level for bacteria and provides completely mixed conditions. Fluidised bed reactors are used to cleanup groundwaters with temperatures in the range 30 to 39 °C (Ahmadvand *et al.*, 1995; Gandee *et al.*, 1995) and are also able to treat groundwater with lower temperatures, *ca* 20 to 29 °C (Jarvinen *et al.*, 1994; Puhakka and Melin, 1998).

A recent modification is the use of granular activated carbon (GAC) reactors. These have the advantage of establishing rapid microbial growth due to sorption of contaminants on the granular activated carbon (Edwards *et al.*, 1994; Sutton and Mirsha, 1994).

1.9.2 Upflow fixed-film reactor

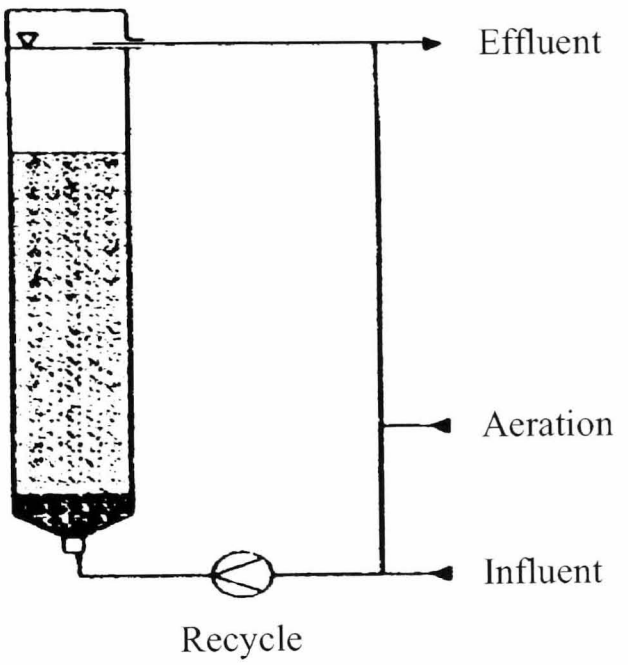


Figure 1.3 Generalised schematic of an upflow fixed-film reactor.

In an upflow fixed-film reactor (Figure 1.3), bacteria are growing on submerged inert packing. The upflowing water provides microorganisms in the biofilm with substrates and nutrients. Under aerobic conditions oxygen required is supplied through the use of air sparging. These types of reactors are used for the clean up of contaminated groundwaters. The use of granular activated carbon as packing provides benefits for microbial growth due to sorption of contaminants on the granular activated carbon (Weber *et al.*, 1970; Bouwer and McCarty, 1982)

1.10 Nitrification and denitrification

Originally, activated sludge system design was only concerned with the removal of organic carbon substances from wastewaters. During the past two decades however, more stringent effluent standards for nutrients (nitrogen N and phosphorus P) imposed by legislation have in many countries led to the development of more complicated activated sludge process configurations specifically designed to achieve biological nutrient removal. A general characteristic of all systems is that mixed liquor is cycling through aerobic, anoxic and anaerobic conditions to obtain nitrification, denitrification and biological phosphorus removal (Randall, 1992).

Nitrogen N is an essential nutrient for all organisms, being present in the form of proteins, nucleic acids, adenosine phosphates, pyridine nucleotides and various pigments. Fish expel various nitrogenous waste products through gill diffusion, gill cation ion exchange, urine and faeces. In aquaculture, uneaten food also contributes to nitrogenous waste loading. However, a major source of nitrogen pollution of surface waters is urban wastewaters (Mateju *et al.*, 1992).

Both un-ionised ammonia and nitrite (NO_2^-) are toxic to fish at low concentrations. Acute toxicity can occur at 0.2 mg/l NH_3 in salmonids and a maximum permissible level of just 0.002 mg/l NH_3 has been recommended (Haywood, 1983). Nitrite toxicity varies greatly between species and life stages, but a concentration as low as 1.8 mg/l NO_2^- has been documented to be lethal to rainbow trout (*Oncorhynchus mykiss*) within 24 hours (Smith and Williams, 1974). The lethal concentration of nitrate, on the other hand, is an astounding 6200 mg/l for channel catfish (*Ictalurus punctatus*) (Colt and Tchobanolous, 1976), and this toxicity may be due to the high concentration of the corresponding anions (Colt and Armstrong, 1981). By comparison the permissible nitrate level in potable water is more than two orders of magnitude lower (Addiscot *et al.* 1991).

1.10.1 Forms of nitrogen

Table 1.2 lists the oxidation states that elemental nitrogen may assume together with the corresponding inorganic species.

Table 1.2 Simple forms of inorganic nitrogen and the oxidation state of the N atom.

Species	Common name	N
NH_3	anhydrous or free or unionised ammonia, ammonia gas	-3
NH_4^+	ammonium, ammonium ion, ionised ammonia	-3
NH_4OH	ammonium hydroxide	-3
N_2H_4	hydrazine	-2
$N_2H_5^+$	hydrazinum ion	-2
N_2H_5OH	hydrazine hydrate	-2
NH_2OH	hydroxylamine	-1
HN_3	hydrogen azide, hydrazoic acid	-⅓
N_3^-	azide ion	-⅓
N_2	nitrogen, nitrogen gas, dinitrogen gas	0
N_2O	nitrous oxide, nitrous oxide gas, laughing gas	+1
$H_2N_2O_2$	hyponitrous acid	+1
NO	nitric oxide, nitric oxide gas	+2
NO_2^-	nitrite ion	+3
HNO_2	nitrous acid	+3
N_2O_3	dinitrogen trioxide, nitrogen sesquioxide, anhydrous nitrous acid	+3
NO_2	nitrogen dioxide	+4
N_2O_4	dinitrogen tetroxide, nitrogen peroxide	+4
NO_3^-	nitrate ion	+5
HNO_3	nitric acid	+5
N_2O_5	dinitrogen pentoxide, anhydrous nitric acid	+5

1.10.2 Biological nitrification

Biological nitrogen (N) removal requires three reaction steps. In a first phase, hydrolysis and degradation of nitrogen-containing organic compounds release ammonium nitrogen (NH_4^+–N). In a second aerobic step, nitrification or the biological oxidation of NH_4^+ to nitrite (NO_2^-) and nitrate (NO_3^-) takes place, by NH_4^+ and NO_2^- oxidising bacteria respectively.

There are two phylogenetically distinct groups of bacteria that collectively perform nitrification. Ammonia-oxidising bacteria (or nitrite bacteria) obtain energy by catabolising unionised ammonia to nitrite (Suzuki *et al.*, 1974), while nitrite-oxidising bacteria (or nitrate bacteria) mineralise nitrite to nitrate. The former process is sometimes termed nitritation, the latter nitratation.

Bacteria of the genera *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* participate in the first stage of nitrification. The second stage involves the genera *Nitrobacter*, *Nitrococcus*, *Nitrospira* and *Nitrospina,* (Watson, 1971; Watson *et al.*, 1986; Meincke *et al.*, 1989). All members of the *Nitrobacteraceae* family are gram-negative chemoautotrophs, also known as lithotrophs. Chemoautotrophic bacteria are characterised by their ability to utilise an inorganic chemical substrate (*e.g.*, NH_3, H_2, Fe^{2+}) as a source of electrons for the immobilisation of inorganic carbon (*i.e.*, CO_2(aq) or HCO_3^-) into new cell material (or biomass). Chemoautotrophs are aerobic, usually employing dioxygen gas (O_2) as the final or terminal electron acceptor.

1.10.3 Biological denitrification

Biological denitrification is a respiratory process involving the sequential reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and finally to dinitrogen gas (N_2) (Payne, 1973).

As stated biological nitrogen (N) removal is a three step process, the third step or anoxic phase being denitrification. Oxidised nitrogen species serve as electron acceptors and are converted to nitrogen gas (N_2) that escapes from the system. In contrast to nitrification, which lowers the pH, the denitrification step raises the pH.

Ammonia oxidisers are capable of reversing what is considered to be their natural reaction process by reducing nitrite (NO_2^-) even in the presence of oxygen (O_2) (Hooper, 1986). A number of studies have reported that autotrophic nitrifiers are able to reduce NO_2^- to NO, N_2O or N_2 under oxygen limiting conditions (Poth, 1986; Remde and Conrad, 1990; Bock *et al.,* 1995). This process is known as aerobic denitrification with the end product of the reaction being dependent on the organisms and the presence of a suitable electron acceptor (Poth, 1986; Remde and Conrad, 1990; Bock *et al.*, 1995). Although some autotrophic bacteria are able to reduce nitrate by using it as an electron acceptor, most of the nitrate-reducing bacteria are facultative anaerobic heterotrophs. The principal genera are *Pseudomonas*, *Micrococcus*, *Achronobacter* and *Bacillus*.

The ability to denitrify is thought to be a common trait of municipal activated sludge systems. This ability can be utilised for the removal of nitrate or the reduction of aeration costs through the substitution of nitrate for oxygen as the terminal electron acceptor. For an activated sludge to have the trait of denitrification, the organisms with the genetic capacity for denitrification must be present. The genetic capacity must also be translated (via enzyme synthesis) into specific enzymes that catalyse the reactions of denitrification. In addition, the enzymes must be active under the given environmental conditions.

1.11 Inhibition of the nitrification/denitrification processes

Biological removal of nitrogen from wastewaters is a major process operated by sewage treatment works. In this process nitrogen as in ammonia is transformed to nitrite by *Nitrosomonas* sp. followed by conversion to nitrate by *Nitrobacter* sp. and finally through to nitrogen gas. The nitrification process is sensitive to environmental factors such as temperature, dissolved oxygen levels, pH, available substrate and inhibition due to the presence of organic compounds, sludge age and growth (selective wash out of specific bacterial strains), and the build up of metabolites which may exert toxic effects at high concentrations.

1.11.1 Growth

The growth of nitrifying bacteria is slow even under optimal conditions. Most species of nitrifying bacteria grow optimally between 25 °C and 30 °C, pH 7.5–8.0 and ammonia concentrations between 2 and 10 mM or nitrite concentrations between 2 and 30 mM for ammonia and nitrite oxidisers respectively (Bock *et al.*, 1986). Under these conditions the generation time is typically around eight hours for *Nitrosomonas* and ten hours for *Nitrobacter* (Bock *et al.*, 1986). Growth and the activity of both *Nitrosomonas* and *Nitrobacter* are not optimal at the same oxygen level. Growth of both types of organisms is inhibited at high oxygen concentrations despite respiration being increased (Bock *et al.*, 1986; Prosser, 1989). The optimal dissolved oxygen (DO) concentration for growth of ammonia and nitrite oxidisers is in the region of 3–4 mg/l O_2 (Prosser, 1989).

According to the studies of Anthonisen *et al.* (1976), the non-ionised forms of ammonium and nitrite have – as ammonia (NH_3) and nitrous acid (HNO_2) – an inhibiting effect on both the *Nitrosomonas* and the *Nitrobacter*. The *Nitrobacter* react more sensitively so that concentration of NH_3 in relatively low range is sufficient for inhibition. Abeling and Seyfried (1992) reported that a concentration of 1–5 mg/l NH_3 inhibited nitratation but not the nitritation.

1.11.2 Sludge wastage

The rate at which sludge is wasted from an activated sludge process, both deliberately from the return activated sludge (RAS) and as suspended solids contained in the effluent, will determine the populations of bacterial species maintained in the sludge floc. As heterotrophic bacteria (those responsible for oxidation of carbonaceous matter) grow much faster than the autotrophic bacteria, they will form a greater proportion of the sludge floc under all circumstances when treating sewage, which has a greater content of organic carbon than ammoniacal nitrogen. To achieve nitrification, the rate of sludge wastage has to be sufficiently low to ensure that adequate numbers of nitrifying bacteria accumulate in the floc.

1.11.3 pH

pH is a very significant factor in the growth of microorganisms. The optimum pH is between 6.8 and 7.4, although a commonly cited optimum pH for nitrifiers is 7.8 (Painter, 1970; Jones and Paskins, 1982). pH is a very important factor controlling growth since it has an effect on the biological system at all levels.

At the fundamental level, pH affects enzymatic activity. All biochemical reactions are catalysed by enzymes, which are proteins. These proteins are affected by changes in pH, which affects their structure and activity. Biological treatment systems are adversely affected by drastic changes in pH. For example, a drop in pH from 8.0 to 6.0 could significantly inhibit the population and reduce plant efficiency.

During the metabolisation of various compounds or class of compounds by microorganisms, a pH change will occur in the treated waters if there is not sufficient

buffering capacity. For example, when sugars in high concentration are broken down into organic acid end products, these depress the pH. It should be noted that the 'natural' buffer which operates in all biological wastewater treatment systems and maintains the pH of the system is the carbonic acid/bicarbonate buffer system. The ability of this system to adjust to changes in pH is measured as 'alkalinity'. This bicarbonate buffering capacity of the system takes time to develop especially when a new system is started up.

1.11.4 Industrial effluents

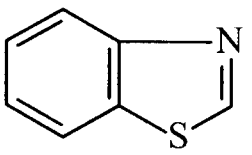
Some materials in industrial effluents inhibit microbial oxidation of ammonia in activated sludge (Downing *et al.*, 1964; Tomlinson *et al.*, 1966). It has been shown by Stafford (1974) and Downing *et al.* (1964) that phenolic compounds have an inhibitory effect on ammonia oxidation (Table 1.3).

Table 1.3 Phenol concentrations producing 75% inhibition of ammonia oxidation.

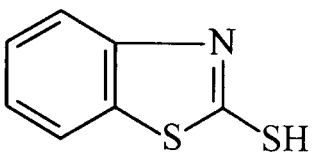
	Concentration of phenol (mg/l) producing 75% inhibition	
	Stafford (1974)	Downing *et al.* (1964)
phenol	5.6	5.6
o-cresol	4.4	12.8
m-cresol	4.0	11.4
p-cresol	5.6	16.5

Rubber chemical additives manufacturing produces wastewaters rich in both inorganic salts and organic compounds. Activated sludge treatment of these wastes is possible but problematic due to the toxicity of some of the thiazoles present. Rada *et al.* (1979) screened a series of benzothiazole derivatives for their virus inhibitory activity and found antiviral activity for mercaptobenzothiazole (MBT, overleaf) with two out of three viruses tested. Not only does MBT exert adverse effects on viruses, yeasts and fungi, it also acts on bacteria. Tomlinson *et al.* (1966) made observations on MBT inhibition of ammonia oxidation and found that 3 mg/l MBT exerted 75% inhibition on the oxidation step.

The compound benzothiazole (BTH) inhibits activated sludge at a concentration of 650 mg/l (Walker, 1989). According to Knapp *et al.* (1982), 7 mg/l BTH causes 50% inhibition and 54 mg/l 100% inhibition of ammonia oxidation.



benzothiazole (BTH)



2-mercaptobenzothiazole (MBT)

Nitrifying bacteria normally constitute only a small percentage of the total biomass in an activated sludge because of their slow growth rate. As a consequence it is not necessary for an inhibiting substance to completely kill them in order to prevent or reduce nitrification, since a relatively small reduction in the growth rate will result in them being washed out of the system.

1.12 The sulphur cycle

All organisms require sulphur for growth. In bacteria sulphur makes up 0.5–1.0% of the cell dry weight and is needed primarily as a component of the amino acids cysteine and methionine. Sulphur also plays an essential role in a variety of enzyme cofactors, including biotin, coenzyme A and coenzyme M, thiamine and lipoic acid, and is critical in many redox processes, both as a building block for iron-sulphur centres and as the redox active component of disulphide bonds. Sulphur is common in the environment, making up about 0.1% of the Earth's crust (Brown, 1982), but much of this material is inaccessible to living organisms. Sulphur for biosynthetic processes is derived from the assimilation of inorganic sulphate by plants and bacteria.

Sulphate is an important stock of sulphur on Earth (Volkov and Rozanov, 1983) and is present as a dissolved compound in seas and oceans (1.3×10^9 megatonne sulphate –S) or as insoluble salt (*e.g.* gypsum layers, 5.0×10^9 megatonne sulphate –S).

1.12.1 The global sulphur cycle

The original pool of sulphur was contained in igneous rocks, largely as pyrite (FeS_2). Volcanic degassing and weathering under aerobic conditions resulted in the transfer of

large quantities of sulphur (S) in the order of 10^{21} moles from igneous rocks to the hydrosphere (freshwater and ocean). The major global reservoirs of sulphur are currently sediments hosting pyrite and evaporites ($CaSO_4.2H_2O$), and in the oceans as sulphate. Geological upheaval caused through events such as earthquakes, result in exposure of rocks with erosion by weather. Pyrite weathering is mainly due to oxidation that produces sulphuric acid, whereas dispersal of gypsum ($CaSO_4.2H_2O$), anhydrite ($CaSO_4$) and other sedimentary sulphate minerals occurs through simple dissolution, the rate of which is dependent on prevailing hydrological conditions. Sulphate is transported back to the ocean by rivers, principally as dissolved sulphate. The residence time of sulphate in the ocean is in the order of 11 million years, based on studies by Schlesinger (1991) and Mackenzie *et al.* (1993).

1.12.2 The atmospheric sulphur cycle

The atmospheric component of the global sulphur stock is highly dynamic with residence times of most gases of the order of a few days, because of rapid oxidation to sulphate. The release of sulphur gases is due to volcanic (mainly SO_2) and biological processes with autotrophic organisms (*e.g.* algae in the oceans, higher plants on land) as well as heterotrophs (bacteria) involved. The dominant gas emitted from terrestrial systems (freshwater wetlands and anaerobic soils) is hydrogen sulphide (H_2S), with dimethyl sulphide (DMS) and carbonyl sulphide (COS) being less important. The ocean is also a significant source of sulphate containing aerosols and of the biogenic gases DMS and COS. The reduced sulphur gases DMS and H_2S are efficiently oxidised to sulphate in the troposphere and are thus returned to the sulphur cycle as inorganic sulphate.

The global geochemical cycle of sulphur has been affected through activities of the growing human population, in particular the ever increasing burning of fossil fuels and the smelting of sulphide ores. Both these activities have led to elevated releases of SO_2 and SO_3 to the atmosphere. Oxidation and hydrolysis of SO_2 causes acidic substances to be formed and are subsequently washed out to land with rainfall. Acid deposition has significantly affected the health of numerous ecosystems and may induce the mobilisation

of aluminium and other potential toxic metals from soils and sediments (Likens *et al.*, 1979).

1.12.3 Sulphur oxidation

Chemically sulphur is one of the most interesting but also one of the more difficult elements; geochemically it is abundant and biochemically it is most important. The complexity of the sulphur cycle originates from the many oxidation states it can assume, as well as from the tendency of sulphur in the zero oxidation state to catenate, forming chains and rings of an astonishing variety.

Table 1.4 Oxidation states of sulphur in common compounds.

Oxidation State	*Compounds*
-2	dihydrogen sulphide H_2S, hydrogen sulphide ion HS^-, sulphide ionS^{2-} as in FeS
-1	disulphane H_2S_2, disulphide S_2^{2-} as in pyrite FeS_2
0	elemental sulphur S_n, organic polysulphanes R–S_n–R
+1	dichlorodisulphane Cl–S–S–Cl
+2	sulphur dichloride SCl_2, sulphoxylate SO_2^{2-}
+3	dithionite $S_2O_4^{2-}$
+4	sulphur dioxide SO_2, sulphite SO_3^{2-}
+5	dithionate $S_2O_6^{2-}$, sulphonate RSO_3^-
+6	sulphur trioxide SO_3, sulphate SO_4^{2-}, peroxosulphate SO_5^{2-}

Bacteria that mediate sulphur oxidations are chemolithotrophs for which reduced sulphur compounds act as energy sources. A generalised reaction mediated by the aerobic bacterium *Thiobacillus thiooxidans* is:

$$HS^- + 2O_2 \longrightarrow SO_4^{2-} + H^+$$

Acid formation is an end product of these reactions, and may lower the pH of the medium to 2 or less.

Overall the *Thiobacillus thiooxidans* reaction is roughly analogous to that mediated by ammonia oxidisers. However, a fundamental distinction between HS^- and NH_4^+ oxidation is that the energetics of the former are such that it may occur under anaerobic conditions.

An example is *Thiobacillus denitrificans*, which can grow as a facultative anaerobe and couple sulphur oxidation to respiratory denitrification. Consequently, HS^- oxidation is not mediated by oxygenases as is CH_4 and NH_4^+ oxidation.

1.12.4 Dissimilatory sulphate reduction

In the microbial sulphur cycle, sulphate reduction into sulphide is mediated by sulphate-reducing bacteria (SRB) via dissimilatory sulphate reduction. This process of bacterial respiration occurs under strictly anaerobic conditions and uses sulphate as the terminal electron acceptor. The SRB are obligate anaerobes that use either H_2 or organic compounds as electron donors and sulphur oxyanions as electron acceptors to produce sulphides, as per the generalised equations below:

$$H_2 + SO_4^{2-} \xrightarrow{\text{chemolithotrophic growth}} H_2O + HS^-$$

$$\text{lactate} + SO_4^{2-} \xrightarrow{\text{chemoorganotrophic growth}} \text{acetate} + CO_2 + HS^- + H_2O$$

All transformations are microbially mediated, and all depend on the oxygen content in the environment.

$$S^0 \text{ in aerated environments} \longrightarrow SO_4^{2-}$$

$$S^0 \text{ in anaerobic environments} \longrightarrow H_2S$$

The H_2S is either released into the environment or it reacts with metals to form the metal sulphide (MS). Both H_2S and FeS are in their most reduced oxidation state, and are oxidised to SO_4^{2-}. Two different metabolic pathways operate in microbes for oxidation.

The first is a two-stage process in which H_2S and FeS are oxidised to elemental sulphur S^0 first (either intracellular *e.g. Phototrophic chromatium*, or extracellular *e.g. Ectothiohodospira chlorobium*) and then to sulphate. The second pathway is mediated by *Thiobacillus*, in which H_2S and FeS are oxidised first to cellular sulphydryl organic compounds, then to sulphides and finally to sulphate. The sulphate produced reacts with water to form sulphuric acid (H_2SO_4). *Thiobacillus thiooxidans* produces huge quantities of sulphuric acid, an acidophile (optimum pH for growth 2.0–3.5). Finally, sulphate can be used as an electron acceptor and reduced to H_2S by sulphate-reducing bacteria (SRB).

1.13 Biological degradation of xenobiotic compounds

Single celled organisms have been on the Earth 2–3 billion years longer than any other life. Bacteria are one of the most diverse life forms on Earth and may consist of more than one million species (American Society for Microbiology, 1994). More conservative estimates suggest the number of bacterial species to be over 110 000 (Hawksworth, 1991b). Only a fraction of these species have been identified and even fewer are being studied or are in culture collections. By one estimate, only 1–10% of the Earth's bacterial species have been identified, leaving a vast portion of that biota unknown and therefore unstudied (Hawksworth, 1991a).

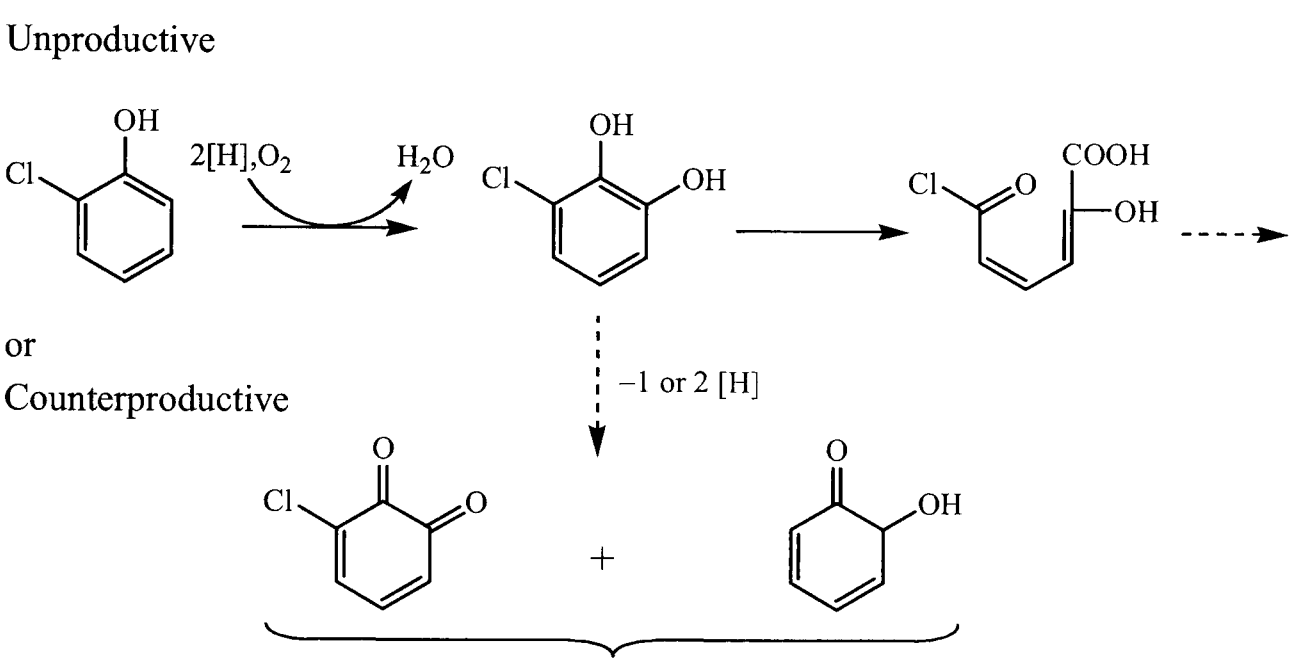
Bacteria are the most numerous of the microbial groups in soils, but because of their small size, 1–10 μm, it is estimated that they account for less than half the total biomass in agricultural soils (Alexander, 1977). Metabolically bacteria are very diverse and use many different sources of energy and carbon. Traditionally bacteria have been classified according to their principal means of obtaining carbon and energy, *i.e.*, phototrophs, chemotrophs, autotrophs, heterotrophs or lithotrophs (see Section 1.8.1).

Bacteria are important for the breakdown of organic material and nutrient cycling. Most natural and xenobiotic compounds can be broken down by bacteria with few compounds proving recalcitrant (Mainprize *et al.*, 1976). However many compounds, particularly the more complex, require more than one type of bacteria.

The fate of environmental pollutants is largely determined by abiotic processes such as photooxidation, or metabolic activities of microorganisms. Since catabolic enzymes are not totally specific, they can act on more than their natural substrate. This explains why the majority of xenobiotics are subject to fortuitous metabolism (they are said to be co-metabolised). Normally, an initial catabolic enzyme or sequence of enzymes convert a chemical to an organic product that is not further metabolised. Thus, for a co-metabolically active organism, the process is unproductive because it is not coupled to energy conservation. Therefore, in a natural mixed population, a wide variety of dead-end products may be accumulated and subject to physical and chemical secondary reactions. These may build up to a level where they exert toxic effects resulting in inhibition of the

biomass. The metabolites themselves are subject to further metabolisation producing daughter metabolites, as a consequence of which it becomes difficult to determine exact metabolic pathways when using a mixed culture.

Since initial oxidation by mono- or dioxygenases requires reduction equivalents or the loss of active protein by suicide inactivation, co-metabolism may even be counterproductive. An example is the co-oxidation of 2-chlorophenol by phenol- or cresol-degrading microorganisms, which gives rise to the accumulation of 3-chlorocatechol (Schimdt *et al.*, 1983).



The latter, if subject to *meta*-cleavage, generates an aryl chloride, which irreversibly inactivates the ring cleavage enzyme (Bartles *et al.*, 1984). As a consequence, chlorocatechols accumulate, which by autoxidation generate highly reactive products such as quinones or phenoxyl radicals. With increasing complexity of a xenobiotic, it would not be expected that complete catabolic pathways would be found in a single organism.

Besides incomplete oxidation and accumulation of dead-end metabolites, a higher degree of biodegradation and even mineralisation can be expected when co-metabolic activities within a microbial community complement each other. Such syntrophic interactions actually exist and in certain cases the concerted action of a two-species culture is well understood. A case in point is the degradation of naphthalenesulphonates which are structural features and building blocks of azo dyes. *Sphingomonas* strain BN6 harbours the

ability to degrade naphthalene-2-sulphonate (2NS) to salicylate. Because the latter compound is not further degraded and is toxic to strain BN6, it can grow with 2NS only in the presence of a salicylate assimilating organism (Nortemann *et al.*, 1986).

Bacteria impact ecosystems through a large list of functions distributed amongst a large number of different trophic groups. Some bacteria have the potential for dinitrogen fixation (Sprent, 1979) or methane production or utilisation (Jones, 1991). In the nitrogen cycle, several bacterial species are involved in ammonification and other nitrogen (N) transformations are accomplished by selected species. Chemo-autotrophic nitrification is accomplished by a limited number of genera of aerobic autotrophic bacteria (Bielser and Schmidt, 1978; Bock *et al.* 1989), whereas heterotrophic nitrification is conducted by a broader group of organisms. Denitrification involves a variety of facultative and obligate anaerobic bacterial species (Tiedje *et al.*, 1984). Other levels in the nitrogen cycle, such as dissimilatory NO_3 and NO_2 reductions, are performed by a few species from widely distributed genera.

In the sulphur cycle, only a few genera within a narrow range of environmental conditions are responsible for the transformations. Sulphate reduction involves a variety of facultative and obligate anaerobic bacteria (Tiedje *et al.*, 1984). Sulphur oxidation, on the other hand, is the result of the activity of a limited number of genera of autotrophic bacteria (Bielser and Schmidt, 1978; Bock *et al.*, 1989). Over fifty species of methanotrophs are responsible for the production or catabolism of methane (Jones, 1991).

While there is a huge amount of knowledge regarding the biological processes that occur during the biological breakdown of organic chemicals (xenobiotics) in various ecosystems, relatively little is known about the specific metabolic pathways which occur. It is the objective of this research to determine the nature of how some specific organic compounds are transformed during biological removal from industrial wastewaters.

1.14 Activated sludge

The practice of utilising mixed culture microorganisms to perform biologically mediated chemical transformations has a long history in the brewing, pharmaceutical and dairy

industries. These microorganisms have also long been used in the treatment of wastewaters from both municipal and industrial sources.

In the treatment of wastewater, microorganisms (mainly bacteria) utilise the soluble organic matter present in the waste stream as a source of food. The organic components present are consumed by the bacteria during which they are converted into carbon dioxide, water and also energy to produce new cell material. The soluble pollutants are finally converted into insoluble biomass, which is then removed mechanically from the system in the form of waste activated sludge (WAS). The Flexsys plant at Ruabon (see Section 1.15 below) for instance wasted about 5% sludge per day. Sludge disposal can be by a variety of methods including landfill, incineration, composting and spreading on agricultural land.

In order for the biological system to function properly various parameters need to be constantly monitored and controlled. The more critical of these are mixed liquor suspended solids (MLSS), dissolved oxygen concentration (DO), pH, and nutrient levels (ammoniacal nitrogen and phosphate). Historically, control strategies have concentrated on monitoring and controlling the system parameters with little or no attention being paid to the biomass.

1.14.1 The bacteria

Bacteria are typically 1–2 μm wide and 2–20 μm long. Their size makes it difficult to study them without the use of a high power microscope (×1000+) and staining techniques. A common staining technique applied to the characterisation of bacteria is the 'Gram stain'. In this procedure bacteria are categorised as being either Gram positive or Gram negative, which indicates a fundamental variation in cell-wall structure.

Bacteria are also categorised on the basis of other characteristics such as:

- Use of oxygen in degrading organic compounds
  - use oxygen only – aerobic
  - can metabolise with or without oxygen – facultative
  - do not use oxygen – anaerobic
- Use of carbon sources (organic – heterotrophic, carbon dioxide – autotrophic)

- Optimum growth at different temperatures
  - thermophiles (55 to 75 °C)
  - mesophiles (30 to 45 °C)
  - psychrophiles (0 to 15 °C)
  - obligate (15 to 18 °C)
  - facultative (25 to 30 °C)

In the main, aerobic wastewater treatment plants operate in the temperature range of 10 to 40 °C, and as a consequence contain mainly mesophilic bacteria. These consist of both the Gram positive types, such as *Bacillus* and the Gram negative types such as *Pseudomonas*. The collective term used for these bacteria (microorganisms) is 'biomass'. All these bacteria interact to transform organic material into new biomass, carbon dioxide and water.

This biomass is the 'workforce' of the wastewater treatment system. It is in a constant dynamic state of flux, one microbial species dying while others grow and become more dominant. Under adverse conditions such as toxic shock, certain bacterial populations may be reduced or eliminated, causing a reduction in the overall operating efficiency of the treatment plant. Examples of shock are: accidental release through spillage; introduction of a new compound for which no enzymatic reaction is operable (slow acclimation may occur to generate the required enzymes); and sudden changes in pH or salinity of the system.

This project has involved the investigation of the processes involved in the biodegradation of those compounds that are manufactured at the Flexsys Rubber Chemicals production facility in North Wales.

1.15 Flexsys Rubber Chemicals

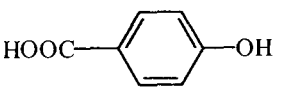
The Flexsys Rubber Chemicals site is built on a steep hillside at Cefn Mawr some 10 miles south of Wrexham and close to the River Dee. The Dee is a drinking water abstracted river, which supplies water to some 5% of the UK population. The plant was originally built by a German chemist, Robert Ferdinand Graesser. He chose the site because of its topography and planned to extract oils and waxes from shale available from neighboring collieries.

Monsanto of USA made a part purchase of the site in 1920, the first acquisition for the American company outside of the United States. Then eight years later Monsanto purchased the remaining part of the company. Due to competition from the U.S. oil industry the process of distillation at the Ruabon site was stopped and the site switched to producing phenol. The site at Ruabon became a major producer of phenol along with other chemicals including saccharin, vanillin, salicylic acid and aspirin.

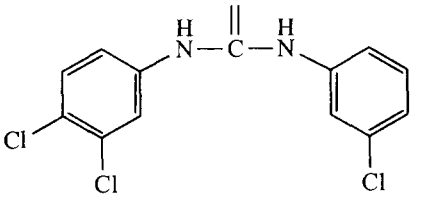
During the early 1930s the company began production of rubber chemicals, the range of products manufactured continuing to increase up to the late 50s and early 60s, along with other chemical products. In 1996 Monsanto and Akzo Nobel pooled their rubber chemical manufacturing businesses and formed a joint venture company 'Flexsys Rubber Chemicals'. Products manufactured at the Flexsys Ruabon site can be categorised as shown below.

Fine Organics

para-hydroxybenzoic acid (PHBA)



trichlorocarbanilide

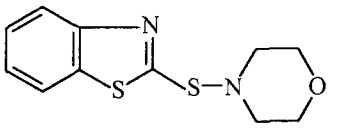


Rubber Chemicals

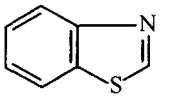
The products in this group can be split into three groups (or classes).

1 Accelerators

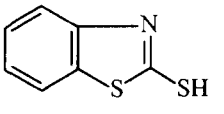
2-morpholinobenzothiazole



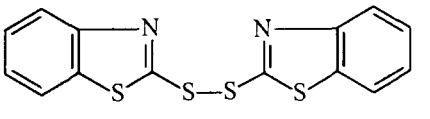
benzothiazole



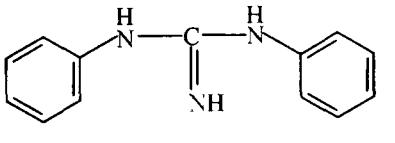
2-mercaptobenzothiazole



bis(2-benzothiazolyl) disulphide
(2,2′-dithiobisbenzothiazole)

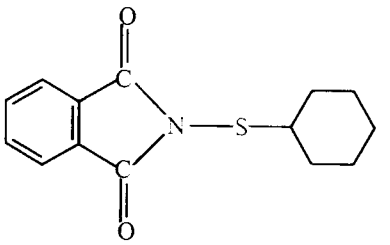


diphenylguanidine



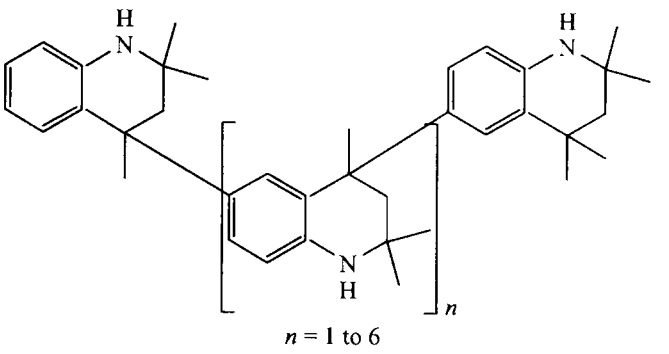
2 Retarders

PVI (prevulcanisation inhibitor)
(cyclohexylthiophthalimide)



3 Antidegradants (Antioxidants)

Flectol TMQ
Poly(1,2-dihydro-2,2,4-trimethylquinoline)



Functional Products

Syton colloidal silica solution

The processes used to manufacture the above range of products all generate wastewater, which has to be treated prior to discharge to the River Dee. Effluents on the site are classed as either strong effluents – those from sources such as product washings, filtrate, centrate liquors or spillages – or weak effluents, the source of which is from surface water run off, cooling tower water, or steam ejectors. These two types of wastewater are collected and stored in separate tanks and only mixed prior to feeding onto the treatment plant.

Historically, the effluent from the site was treated in the following manner. Weak liquors were collected in large reservoirs and released under controlled conditions to the Trefynant Brook, a natural watercourse. Strong liquors were run to soak-away pits.

Due to the variations in the volume flow of the River Dee, especially during the summer period, storage of waste liquors was considered. Two 2.5 million gallon lagoons were built on the river bank during 1945 and were popularly referred to as the 'ponds'. The release of liquor to the river was conducted under controlled conditions from these two lagoons. All the strong liquor was disposed of in this way until 1988, the weak liquor continuing to be run to the brook as before.

Continued expansion of the site soon began to generate waste liquors at a rate that exceeded the 'ponds' capacity. It became obvious that some form of pretreatment to reduce the effluent load by other means before discharging to the river would have to be introduced.

In 1948, the first stage of a biological treatment plant was installed. This plant consisted of strong liquor balancing tanks, a mixing chamber, a lime slurry feeder (for pH control), a clarifier, two biological percolating filters and sludge drying beds.

Continued expansion of the site during 1954–1956 resulted in the treatment plant not being able to handle the increased load. Following a court injunction during 1954–1956, the treatment plant was expanded with the addition of a second stage biological reactor.

Over the years the plant was expanded and modified to what is in operation today.* The present plant makes use of the patented Vitox process developed by BOC (British Oxygen Company) that involves a pure oxygen injection system (see Section 1.8.7). The plant currently uses two 2 400 m^3 activated sludge biological reactors to treat the site effluent prior to discharge to the River Dee.

This plant produces an effluent discharge with the following typical characteristics:

- residual ammonia in the range 1–10 mg/l;
- residual TOC (total organic carbon) 28–35 mg/l;
- colour pale yellow to deep amber (however on occasion the colour is a deep red);
- pH normally in the range 6.8–7.3.

One of the major problems associated with the operation of the wastewater treatment plant at Ruabon is that of the variation in the influent matrix to the biological reactors. The manufacturing plants are run to meet various production schedules. These schedules make allowances for planned shutdowns to complete any maintenance work but do not take account of unplanned breakdowns or process problems. During these shutdowns the

*This thesis is written as if the wastewater treatment plant were still operational. However, shortly after the experimental work for this project had been completed and the first draft of the thesis had been finished, Flexsys Rubber Chemicals ceased production at Ruabon.

effluent from one or more plants will not be present in the total influent to the wastewater treatment plant. An even greater problem is coping with an uncontrolled or accidental release (*i.e.* spills). In short, the wastewater treatment plant, if considered to be a manufacturing unit whose product is good quality water for discharge to the local watercourse, is unique in that it is the only process which has little or no control over the quality or quantity of its incoming raw materials.

The overall aim of this research is to develop a clearer picture of the chemical processes operating within the biological reactors by investigating the metabolic pathways for the breakdown of the various products manufactured at Ruabon. One specific objective is to identify conditions that give rise to the formation of the red colour in the effluent and if possible to identify the substance containing the red chromophore.

To undertake the above study a series of small pilot plant biological reactors were built, each consisting of a 4 litre polycarbonate plastic reactor and a 2 litre settler unit made of the same material. The feed was supplied from a 25 litre glass aspirator using a Watson-Marlow peristaltic pump, the treated waters from the system being extracted subsurface from the upper layer of the settler using a second Watson-Marlow peristaltic pump. A third peristaltic pump was used to recycle sludge from the bottom of the settler (RAS – return activated sludge), back into the main reactor unit. Dissolved oxygen was maintained at a level between 1.0–2.5 mg/l by use of small air pumps connected to glass sintered sparging tubes (porosity 0) to generate a fine stream of small air bubbles for maximum gas transfer to the surrounding medium.

These laboratory units modeled most of the main plant characteristics. However, there are certain aspects which cannot be modeled these being the use of pure oxygen, the large surface area of both settler / biological reactors, and the front end clarifier and sludge thickener. However, the large 25 litre glass feed aspirators allow settling of any solids due to the quiescent state within the aspirators, which results in them performing in a similar manner to the main treatment plant primary clarifiers.

These units were used to study the effects of changes in pH, dissolved oxygen, hydraulic and organic loading on metabolic performance as well as looking at specific effects due to the presence of higher than normal levels of organic compounds such as those manufactured at the Ruabon site.

One key and fundamental element of this research is that it is based on continuous feed to the bioreactors and thus mirrors the conditions more closely than static batch models do, when compared to the results obtained from a full scale treatment plant. The volume flows and reactor sizes are all scaled to the main plant and so reaction times and results closely resemble those of the main treatment plant.

The majority of literature references cited relate to fed-batch static models where the compound(s) under investigation are dosed to the biomass at a fixed concentration and then biomass reaction under these conditions is studied. The reality is that toxic influents are changing with time and concentration, and this is the basis on which this study is based.

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CHAPTER 2
EXPERIMENTAL PROCEDURES

2.1 The laboratory model

As outlined in Chapter 1, in order to study how the biomass or 'activated sludge' system reacts when operating conditions are changed, a laboratory model representing the main wastewater treatment plant was designed and built in the laboratories at Flexsys Rubber Chemicals. This model consisted of three identical units, each of which was made up as follows. A twenty-five litre glass aspirator was used as the primary clarifier and feed tank. This unit was not stirred, unlike the primary clarifier on the main treatment plant which is slowly stirred. The laboratory model bioreactor consisted of a four litre polycarbonate vessel with an overflow outlet feeding into a two litre settler unit (also constructed out of polycarbonate). The treated effluent was withdrawn from the surface of the settler unit and pumped into a twenty-five litre polythene carboy. Various multiple head Watson Marlow peristaltic pumps were used to control the feed rates, return activated sludge (RAS) rate and effluent removal. The dissolved oxygen (DO) levels were maintained by a supply of air pumped into the reactor through porosity 0 sintered gas sparging dip tubes, using small diaphragm air pumps. Figure 2.1 shows a schematic of the laboratory model.

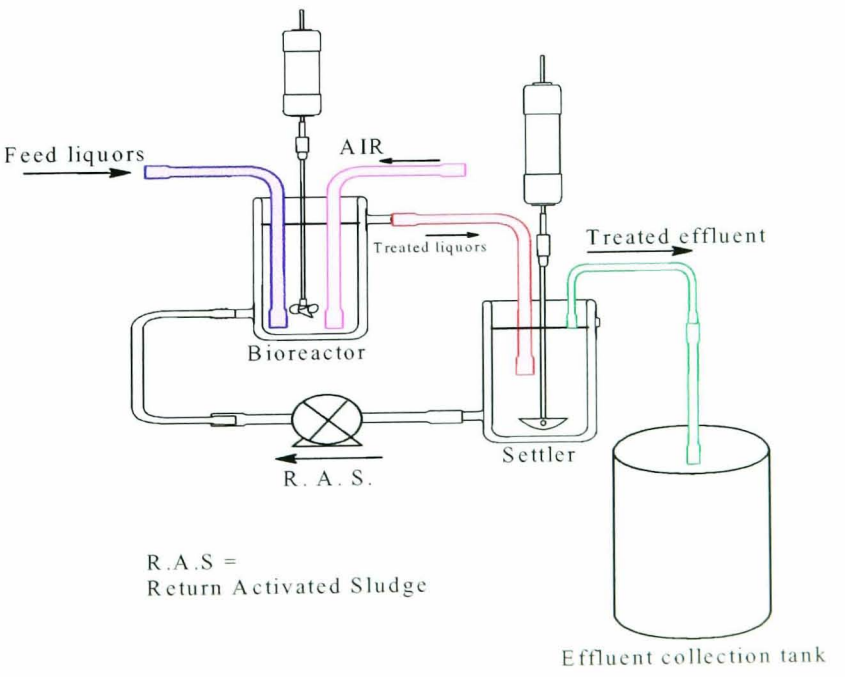


Figure 2.1 Laboratory biological treatment model.

2.1.1 Laboratory model operation

Once the units had been physically built and located in the laboratory, the first step was to leak test the model reactors and check the operation of the various pumps and air supply system. Following successful leak testing the system was primed with sludge from the main wastewater treatment plant. This sludge had a long history of treating effluent from the various rubber chemicals manufacturing plants at Ruabon. Priming of the settler unit was achieved through use of final effluent rather than water (this ensured the maintenance of system salinity and pH).

The system thus primed was then allowed to equilibrate while being fed with 'normal' feed taken from the main wastewater treatment plant. During this equilibration period, flow rates were monitored for the feed, return activated sludge (RAS) and sampling of effluent from the settler. The typical operating rates were determined for the main treatment plant and these were scaled to the laboratory model as follows.

Calculation of main treatment plant flow rates:

Volume of biological reactor 2400 m^3

Flow = 2400 m^3/day

= (2400 × 1000)/(24 × 60) l/min

= 1667 l/min = 27.8 l/s

Equivalent flow for laboratory model calculated thus:

Volume of laboratory biological reactor 4 l

$\therefore$ flow required to produce one volume change per day is given by:

Flow = (1667 × 4)/(2400 × 1000) l/min

= 2.78 cm^3/min

The main treatment plant operates with a return activated sludge (RAS) rate of one times feed rate *i.e.* feed rate 27.8 l/s, RAS rate 27.8 l/s. The laboratory model was therefore set to a RAS rate of 2.78 cm^3/min to match the main plant. Effluent withdrawal was set to maintain a specified level within the settler unit.

Once all the various flows were set, the model units were started up to allow equilibration of the biological reactors and the settler units. During this time the system was monitored

for steady state operation. Running the model at a RAS rate of 2.78 cm^3/min was found to cause blocking of the RAS line outlet at the bottom of the settler unit. The main plant settler design is a squat wide diameter tank with a conical bottom whereas the laboratory unit is a tall cylinder with parallel sides and a flat bottom. A RAS rate of 2.78 cm^3/min did not allow the laboratory settler unit to function properly. At this flow rate a sludge blanket of almost 50% of the settler volume soon developed, which exerted a significant pressure causing compression of the sludge that resulted in blocking of the outlet. To prevent this a higher RAS rate had to be used: initially a rate of 13 cm^3/min RAS was tried, but ultimately a rate of 5 cm^3/min was found to be sufficient to prevent the sludge blanket building up and blocking the outlet. At this RAS rate a sludge blanket of around 10% settler volume was maintained without any further blockages developing.

During this period of equilibration, samples were taken of the main treatment plant bioreactor to determine typical dissolved oxygen, salinity levels, pH and organic loading in terms of total organic carbon (TOC) and nitrogen in the form of ammonia. This feed analysis data was later used in the calculation of a composition for a synthetic feed to be used in place of the 'Vitox' feed. Typical analysis values for the main plant biological reactor are shown in Table 2.1.

Table 2.1 Analysis values for the main plant.

Salinity
Sodium chloride: average 4700 mg/l, expressed as chloride 2852 mg/l Potassium chloride: average 460 mg/l, expressed as chloride 219 mg/l Sodium sulphate: typically 2510 mg/l, expressed as sulphate 1697 mg/l
pH and dissolved oxygen
pH in the range 6.8 – 7.6 Dissolved oxygen levels varied between 1.4 and 2.8 mg/l
Vitox feed
Nitrogen as total nitrogen (TN) typically 75 mg/l (determined as Kjeldahl nitrogen) Organic loading as total organic carbon (TOC) maintained between 500 and 800 mg/l Inorganic salts in the form of sodium and potassium chlorides and sodium sulphate: found to be the same as in the biological reactor

2.1.2 Baseline data acquisition

To determine if all three laboratory reactor units were operating identically, a period of six months baseline data acquisition was carried out during which the various parameters were monitored. Table 2.2 is an analysis matrix for the three units, depicting the analysis regime for the various units. Table 2.2 contains averages for each of the analysis parameters during the period of baseline data acquisition. Appendix 2A contains full analytical data in tabular form for all parameters monitored.

Table 2.2 Analysis matrix for laboratory model.

		Vitox Feed	BioR1	BioR2	BioR3
Bioreactor analysis	pH	W	D	D	D
	Temp °C		D	D	D
	DO mg/l		D	D	D
	Alkalinity (as $CaCO_3$) mg/l		D	D	D
	MLSS mg/l		W	W	W
	TKN mg/l	W	D	D	D
Bioreactor Effluent analysis	Colour @ 510 nm 2 cm cell AU		D	D	D
	Ammonia mg/l NH_3–N	W	D	D	D
	Nitrate mg/l		D	D	D
	Chloride mg/l	W	D	D	D
	Sulphate mg/l	W	D	D	D
	TOC mg/l	W	D	D	D
	Aniline µg/l	W	D	D	D
	BTH µg/l	W	D	D	D
	Flectol A µg/l	W	D	D	D
	Dimorpholino ketone µg/l	W	D	D	D

KEY: D = sampled and analysed on a daily basis; W = sampled and analysed on a weekly basis (the feed analysed each time a new batch is obtained from the main WWTP).

Table 2.3 Average analysis results for the baseline data acquisition exercise.

		Vitox Feed	BioR1	BioR2	BioR3
Bioreactor analysis	pH	10.31	7.88	7.89	7.91
	Temp °C		23.8	23.8	23.9
	DO mg/l		2.4	2.4	2.5
	Alkalinity (as $CaCO_3$) mg/l		631	635	638
	MLSS mg/l		11524	11529	11069
	TKN mg/l	77			
Bioreactor Effluent analysis	Colour @ 510 nm 2 cm cell AU		0.82	0.77	0.77
	Ammonia mg/l NH_3–N		0.23	0.23	0.23
	Nitrate mg/l Nitrogen		6.96	6.59	6.66
	Chloride mg/l	3113	3031	3080	3070
	Sulphate mg/l	1693	1865	1900	1879
	TOC mg/l	739	51.75	51.70	51.70
	Aniline µg/l	75190	0.40	0.44	0.40
	Benzothiazole µg/l	1284	1.01	0.83	0.69
	Flectol A µg/l	667	15.40	16.26	13.92
	Dimorpholino ketone µg/l	ND	44.73	46.26	41.96

KEY: AU = absorbance units; ND = none detected

The data are presented below (Figures 2.2–2.4) in the form of line charts for ease of comparison between the three biological reactor systems. The charts clearly show how closely matched the three systems were after a long period of stable operation.

Figure 2.2 shows clearly that the three biological reactors trend to the same temperature. The average for the period was 23.8, 23.8 and 23.9 °C for reactors R1, R2 and R3 respectively. An interesting point to note is the cyclical nature of the temperature profile; this is not the case with the main water treatment plant. This may be due to the much larger physical mass of the biomass in the main water treatment plant bioreactors.

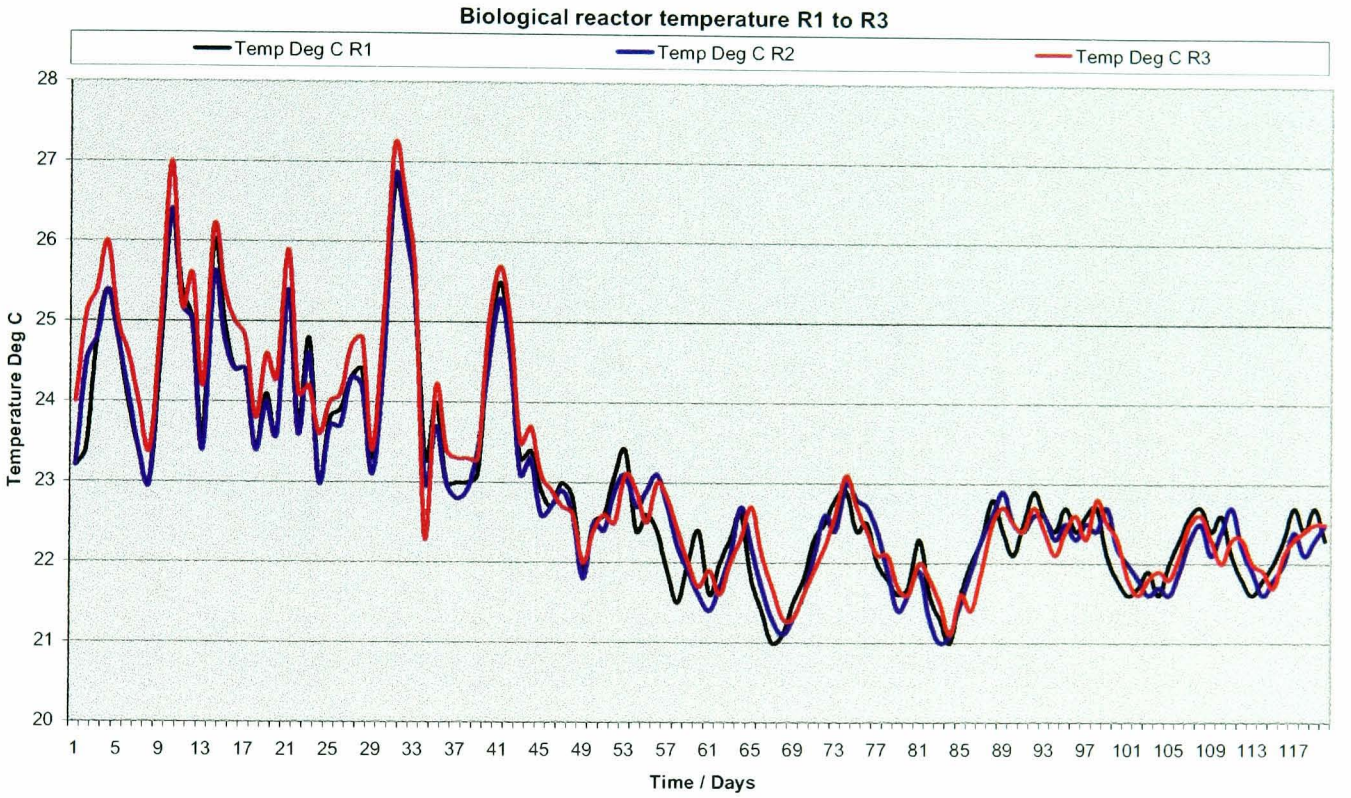


Figure 2.2 Temperature analysis for bioreactors R1, R2 and R3.

The average dissolved oxygen (DO) for the laboratory biological reactors was 2.4, 2.4 and 2.5 mg/l for reactors R1, R2 and R3 respectively (Figure 2.3). By comparison for the same time period the average for the main water treatment plant was 2.0 mg/l. The range for the main water treatment plant was 1.8 to 2.4 mg/l DO; however, the plant has been in operation for a much longer period and the operators of the plant have learned to control this parameter. By comparison the laboratory units had a much wider range of DO initially. This was due mainly to the use of small diaphragm air pumps to supply the oxygen in the form of air. These pumps could supply air at rates up to 800 cm^3/min and were regulated

by means of a simple pinch valve on the air line. The flow rates were set by means of a graduated rotameter allowing the flow to be controlled in steps of 50 cm^3/min increments.

Figure 2.3 clearly shows how the DO levels varied over a wide range during the early days of setting up the reactors. After a period of 33 days control was achieved over the DO content of the system, at an average value of 2.4 mg/l for each of the reactors.

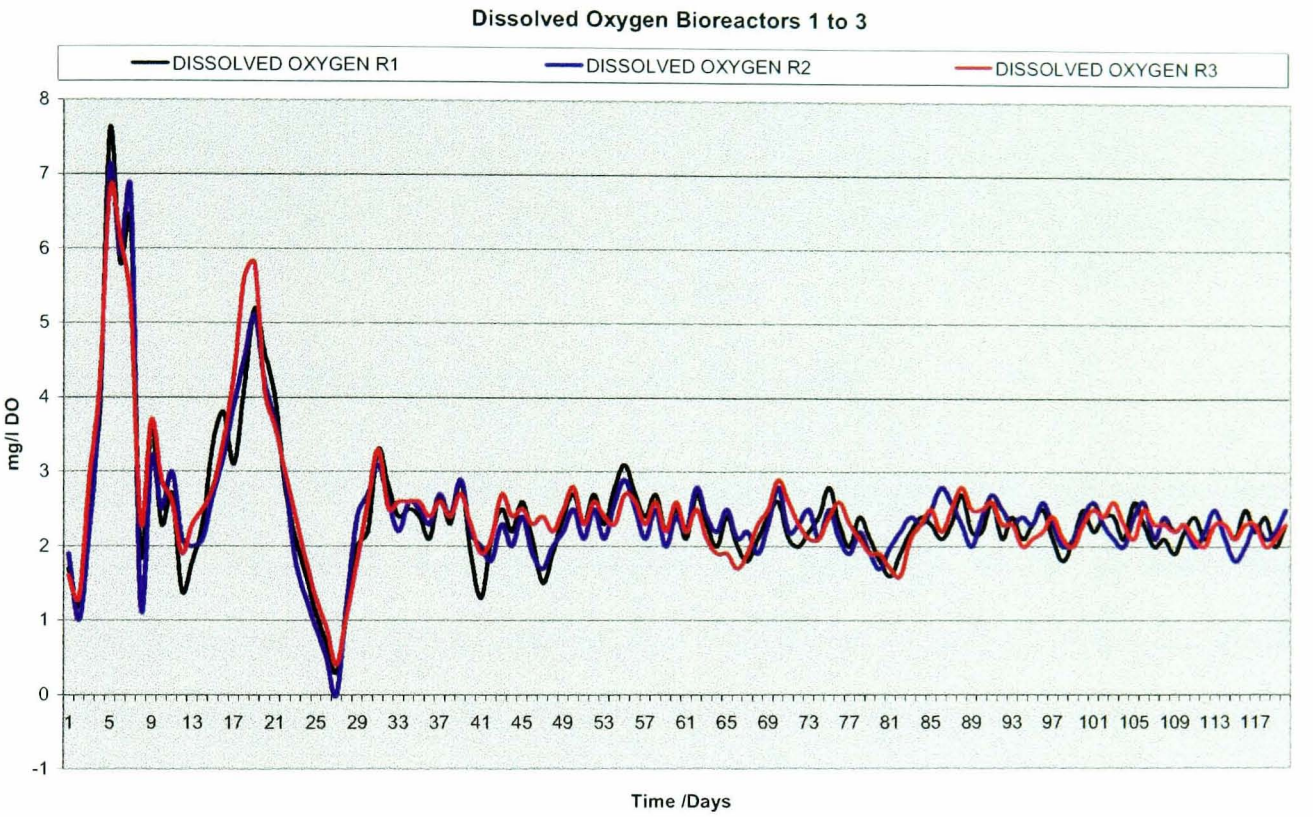


Figure 2.3 Dissolved oxygen analysis for bioreactors R1, R2 and R3.

In the early days of monitoring the total organic carbon (TOC) varied greatly from low values in the 40 mg/l region up to over 140 mg/l. The main water treatment plant by comparison had a mean of 34 mg/l with a range of 17 to 46 mg/l. An explanation for the laboratory model being so different from the main plant may be due to the variance in DO and also the initial shock of relocating the biomass. The TOC for the laboratory units stabilised at 40 mg/l after some 70 days of continuous operation (Figure 2.4 overleaf). The TOC average for the laboratory units was 20 mg/l with a range of 14 to 27 mg/l.

The main conclusion from all the data is that the three biological reactors were operating almost identically and producing values of a similar magnitude to those of the main water treatment plant.

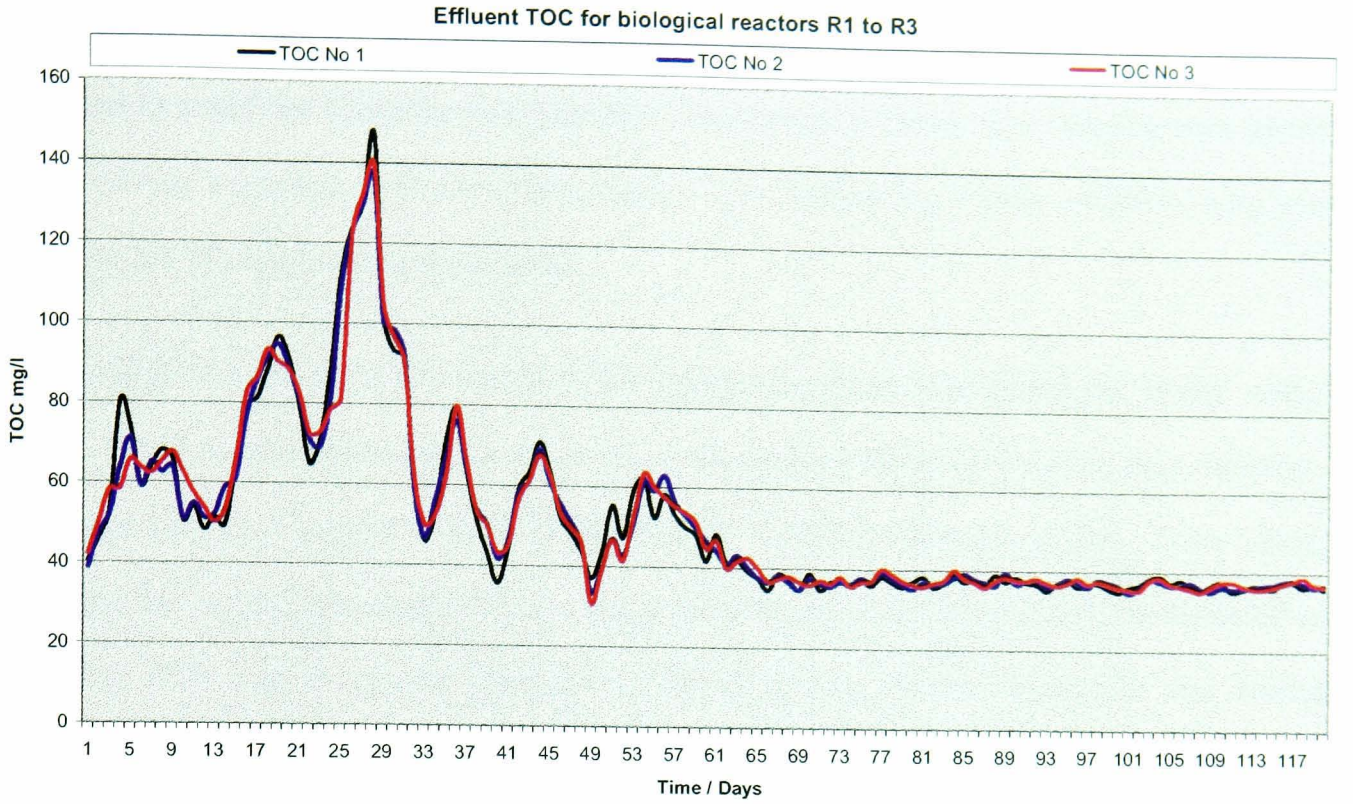


Figure 2.4 TOC analysis for bioreactors R1, R2 and R3.

Once acquisition of these baseline data was complete the reactors were used to carry out an initial study into nitrification/denitrification and the effects of pH on these reactions. Laboratory reactor R1 was designated the control reactor and all operating parameters were kept as constant as possible. Reactors R2 and R3 were subjected to pH changes to see how this in turn affected the level of ammonia in the effluent from the system. This work is discussed in detail in Chapter 3, which deals with inhibition studies.

2.1.3 Preparation of biological reactor R3

The objective of the following procedure was to remove any traces of rubber chemicals and associated metabolites from the biomass, after the initial study was completed into inhibition of nitrification/denitrification. Reactor R3 was set aside and prepared for use in specific metabolic studies. The biomass from this reactor was treated as follows. First a 20 litre volume of a saline solution was prepared by dissolving 106 g sodium chloride, 10.6 g potassium chloride and 50.2 g sodium sulphate in 20 litres of tap water. This produced a solution of similar salinity to that in the main bioreactors of the main water treatment plant and reduced the possibility of cell damage due to sudden changes in environment salinity.

The biomass from reactor R3 was filtered using a Grade 1 Whatman filter paper. The sludge was transferred into a 5 litre plastic beaker and approximately four litres of the

saline solution added. The sludge solution was then gently stirred for five minutes and allowed to stand for a further two minutes. The sludge solution was filtered once again and the exercise repeated a further three times. The remaining saline solution was used to transfer the sludge back to the reactor.

A two litre volume of saline solution was prepared and used to prime the settler unit. This saline solution was based on the analysis of the main water treatment biomass salinity. The prepared biomass was then fed a synthetic feed based on analysis of the 'Vitox' feed, but without the 'rubber chemicals' going onto the main biological reactors, for a period of six months. This was to ensure complete removal of any rubber chemicals and associated metabolites. After two months of being fed this synthetic feed the biomass began to lose its characteristic brown/purple appearance and became very light coloured (a light sandy colour). The final colour of the biomass was almost white and the floc had a light fluffy appearance with excellent settling properties. The colour of effluent from this biological reactor was water white in comparison to reactors R1 and R2 which varied from pale straw to a deep amber colour.

The synthetic feed was prepared in twenty litre batches and fed to reactor R3 at a rate of 2.7 cm^3/min. The composition of this feed is shown in Table 2.4. The quantities given in Table 2.4 were based on analysis of the feed to the main water treatment plant; however, the organic components *i.e.* phenol, acetic acid and ethanol were added to simply make up the organic carbon loading.

Table 2.4 Composition of synthetic feed used for laboratory studies.

Component	*g/20 l*	*mg/l equivalent*
sodium chloride	106.0	5300 (3216 as Cl^-)
potassium chloride	10.6	530 (253 as Cl^-)
sodium sulphate	50.2	2510 (1697 as SO_4^{2-})
ammonium hydroxide	3.76	75 (as N)
phosphorous acid (75% m/m)	1.75	87
phenol	2.67	103
acetic acid (glacial)	6.00	120
ethanol (95%)	19.75	411
The organic mg/l is expressed as TOC	total TOC load 634 mg/l	

No analysis was carried out on the reactor for the first three months, the system was simply allowed to run and acclimate to the new feed matrix. During the final three months a number of parameters were monitored (Tables 2.5 and 2.6) to assess how the biomass was responding to the new feed matrix and to assess if the objective of washing out rubber chemical residues had been successful or not.

Table 2.5 Analysis matrix for bioreactor 3.

Bioreactor Analysis	pH	D
	Temp °C	D
	DO mg/l	D
	Alkalinity (as $CaCO_3$) mg/l	D
	MLSS mg/l	W
Effluent Analysis	Colour @ 510 nm 2 cm path length cell AU	D
	Ammonia mg/l NH_3 –N	D
	Nitrate mg/l Nitrogen	D
	Chloride mg/l	D
	Sulphate mg/l	D
	TOC mg/l	D
	Aniline µg/l	D
	Benzothiazole µg/l	D
	Flectol A µg/l	D
	Dimorpholino ketone µg/l	D

KEY: D = sampled and analysed on a daily basis; W = sampled and analysed on a weekly basis.

Table 2.6 Average analysis, min and max values for bioreactor 3.

		AVE	MIN	MAX
Bioreactor Analysis	pH	7.43	7.34	7.61
	Temp °C	24.4	23.2	25.7
	DO mg/l	2.1	1.5	2.6
	Alkalinity (as $CaCO_3$) mg/l	458	422	476
	MLSS mg/l	18680	18353	20274
Effluent Analysis	Colour @ 510 nm 2 cm path length cell AU	0.07	0.02	0.13
	Ammonia mg/l NH_3 –N	0.23	0.11	0.34
	Nitrate mg/l Nitrogen	0.03	0	0.07
	Chloride mg/l	2309	2221	2456
	Sulphate mg/l	1463	1432	1481
	TOC mg/l	16.48	15.58	17.85
	Aniline µg/l	ND	ND	ND
	Benzothiazole µg/l	ND	ND	ND
	Flectol A µg/l	ND	ND	ND
	Dimorpholino ketone µg/l	ND	ND	ND

KEY: AU = absorbance units; ND = none detected.

All results are presented in full at the end of the chapter in Appendix 2B. A selection of the results are presented in the text in chart form for ease of comparison. Comparison is made between data for the last three months of the reactor R3 being acclimated to the synthetic feed and the last three months of data (a period of stable operation) for reactor R1.

The difference in the two reactor temperature profiles (Figure 2.5) could be attributed to the fact that in reactor R1 the incoming feed matrix is more complex and contains difficult to metabolise components. By contrast reactor R3 is being fed a relatively simple matrix consisting of very simple organic molecules which are far more readily degradable.

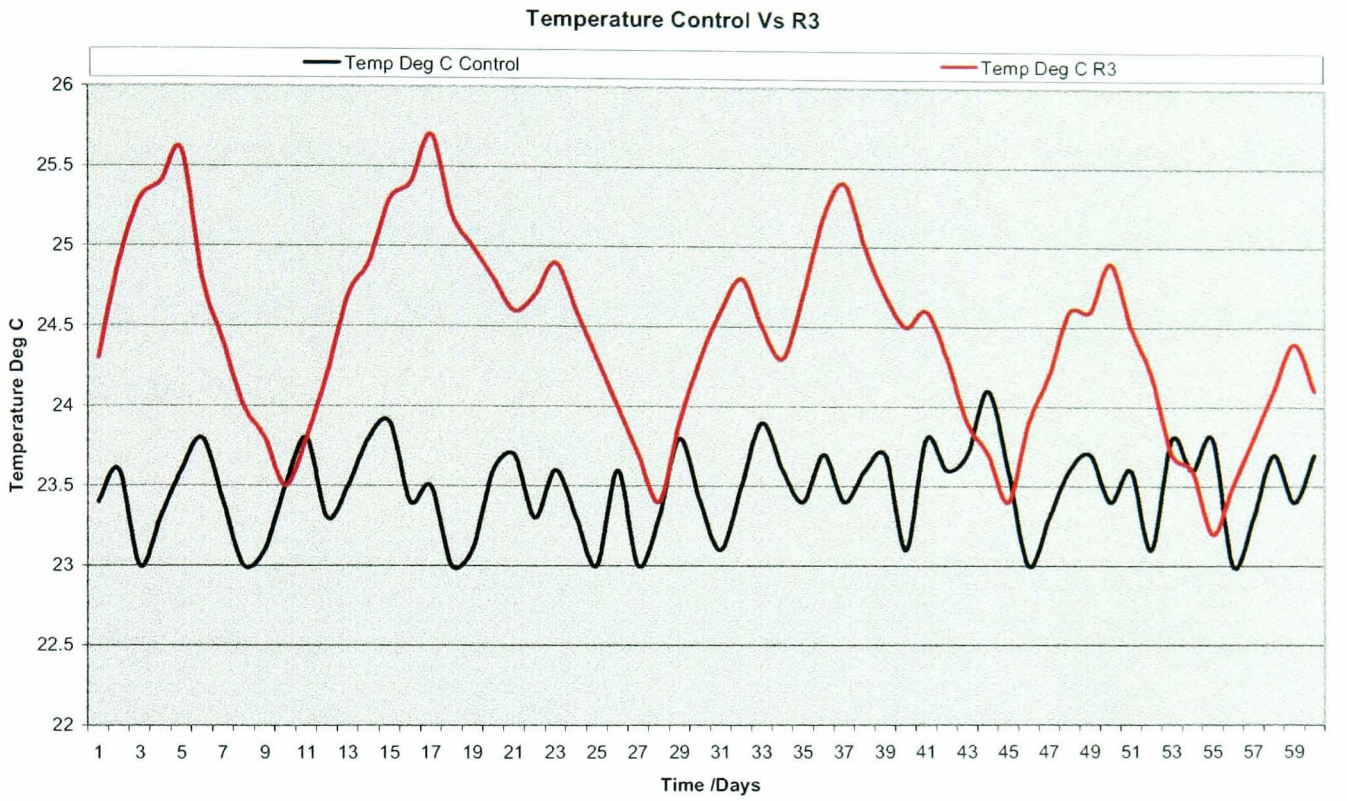


Figure 2.5 Temperature analysis for bioreactors R3 and R1 (control) metabolising a synthetic feed.

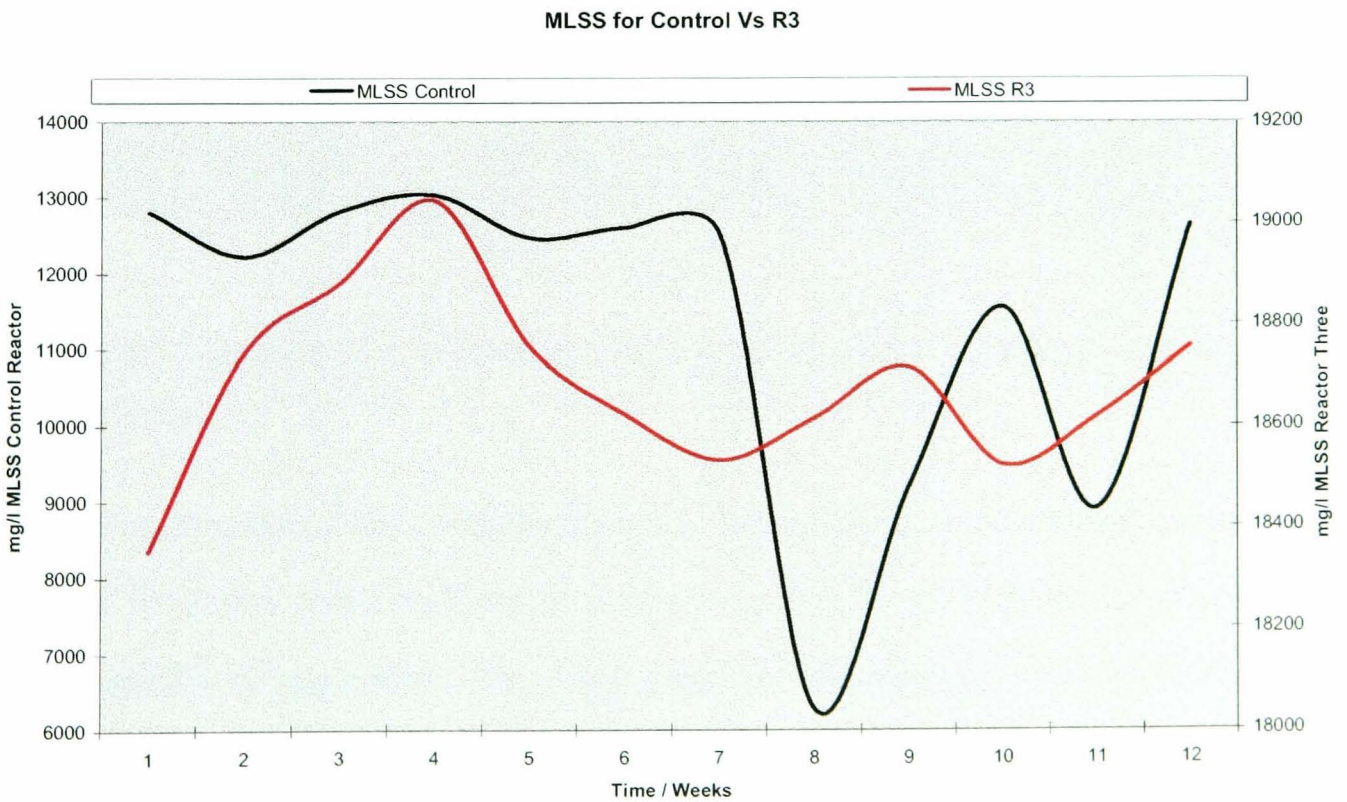


Figure 2.6 MLSS analysis for bioreactors R3 and R1 (control) metabolising a synthetic feed.

MLSS analysis (Figure 2.6) shows how the introduction of a more consistent feed matrix, and one which contains simpler organic compounds that are more readily degradable, gives rise to greater growth within the biomass. The average for all three reactors when being fed a complex matrix of organic compounds was 11 374 mg/l compared to an average for reactor R3 of 18 680 mg/l while being fed this simple and consistent matrix.

Similarly the application of a simpler feed matrix results in a more efficient reduction of input to output TOC (Figure 2.7). For bioreactor R1, with an input TOC value of 634 mg/l, a typical output value during the later part of the baseline data acquisition period was between 35 and 40 mg/l TOC whereas for bioreactor R3 the output TOC was between 15.5 and 18 mg/l TOC.

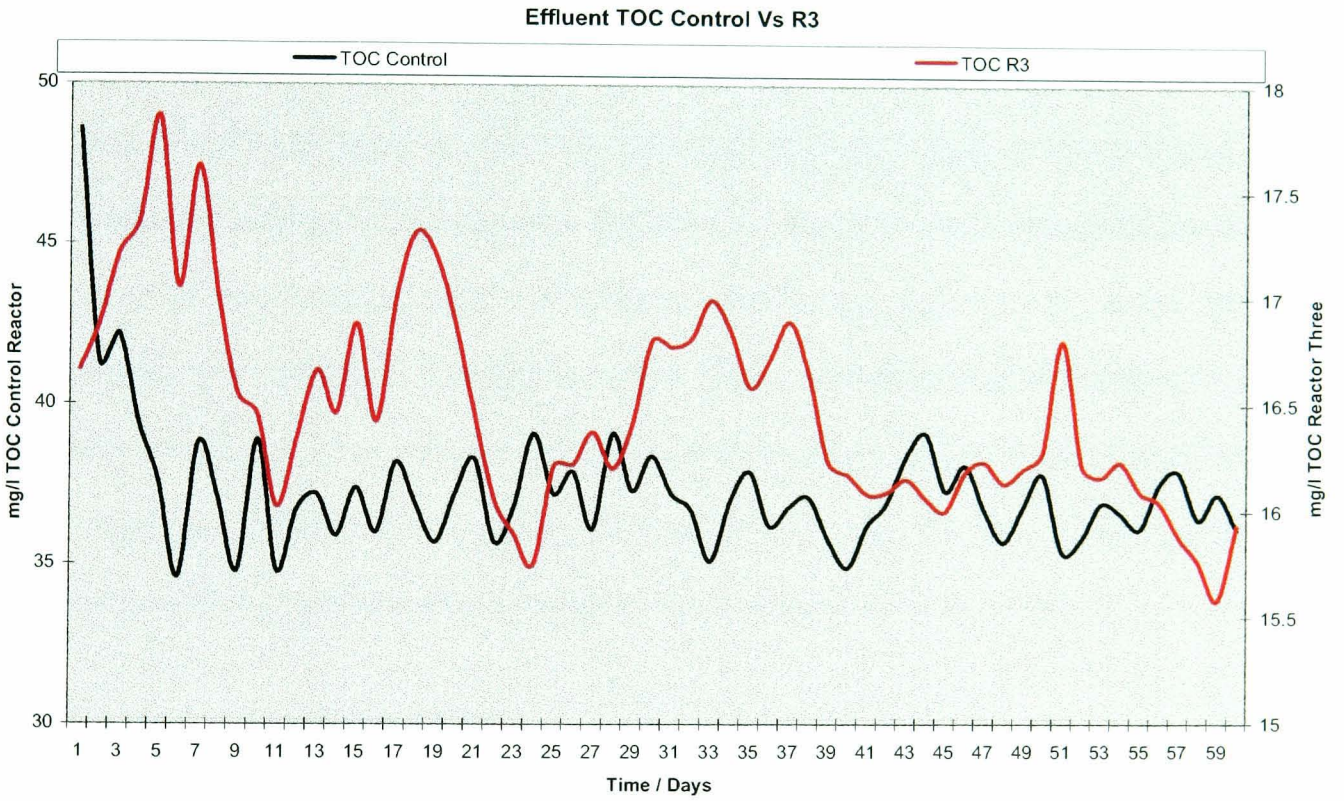


Figure 2.7 Residual TOC analysis for bioreactors R3 and R1 (control) metabolising a synthetic feed.

A significant result of the synthetic feed was the reduction in the effluent colour from the system. This was also coupled with a loss in colour of the biomass which is normally brown/purple in appearance. The final colour of the biomass after acclimation to the synthetic feed was a light sandy colour with an almost colourless effluent. On a few occasions the effluent colour became very slightly coloured (a very pale straw to yellow colour was observed) (Figure 2.8).

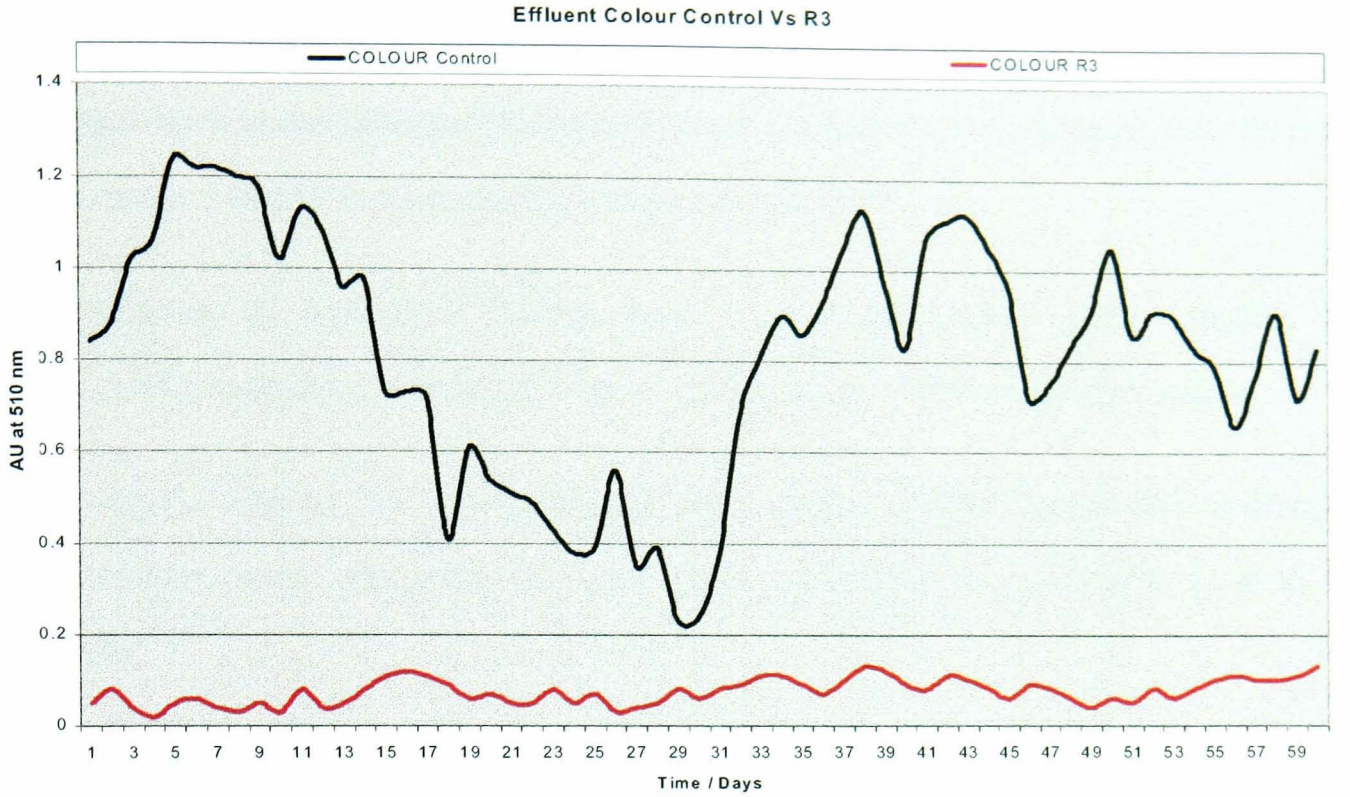


Figure 2.8 Analysis of effluent colour from bioreactors R3 and R1 (control) metabolising a synthetic feed.

Analysis of effluent from reactor R3 confirmed that the washout of all traces of rubber chemicals and associated metabolites had been successful. In all samples examined during the last three months of acclimation to the synthetic feed, no traces of rubber chemicals or metabolites were detected. The biomass thus prepared was then used for specific metabolic pathway studies on how various substrates and substrate mixtures are broken down. This was done by feeding the biomass of reactor R3 with the base matrix plus specific substrates added at various concentrations, typically 25, 50 and 100 mg/l.

2.2 Analytical procedures

All pH measurements were made using a 'HANNA' Aqua Check hand held pH probe. The probe was calibrated weekly using pH 4.0, 7.0 and 10.0 buffer solutions (buffer solutions supplied by BDH Ltd., Poole, Dorset).

The biological reactor alkalinity expressed as calcium carbonate ($CaCO_3$) was determined by titration using standard molar hydrochloric acid and screened methyl orange indicator. Dissolved oxygen readings were taken with a Jenway 9071 DO meter. The meter was calibrated on a monthly basis over two points, using oxygen free water and air to determine the zero and 100% oxygen set points.

Ammoniacal nitrogen determinations were made using an Orion 720A ion meter with the 95-12 gas sensing ammonia probe. The probe and ion meter were calibrated daily over two points, using 5 and 25 mg/l ammoniacal nitrogen solutions.

Determination of biological reactor mixed liquor suspended solids (MLSS) was determined gravimetrically using Whatman 7 cm diameter GF/C grade filter papers.

Total organic carbon (TOC) analysis was performed on a PPM Labtoc carbon analyser. The instrument was calibrated immediately prior to any analysis over the range 40 to 400 mg/l TOC.

Common anion analyses for chlorides, sulphates, nitrites and nitrates were performed using a Dionex DX500 ion chromatograph. The chloride and sulphate determinations used a conductivity detector, whereas nitrite and nitrate were determined using a UV detector at 210 nm. All samples were diluted, 1 in 4, using UHQ water prior to analysis. The chromatographic conditions for anion analysis are shown in Table 2.8.

Table 2.8 Chromatographic conditions for anion analysis.

Instrument	DX500 gradient system with auto injection system
Column	AS14 4 mm × 250 mm (anion separator column)
Conductivity detector	CD20
UV detector	AD20 at 210 nm
Injection volume	50 µl
Eluent	1 mM $NaHCO_3$ / 3.5 mM Na_2CO_3
Eluent flow rate	1.0 cm^3/min

Colour measurements were made using a Cecil model 373 spectrophotometer with 2 cm path length cells, with detection at 510 nm against a blank of UHQ water.

Micropollutant (trace organics) determinations were made using a Varian Saturn 3 GCMS system. This system uses ion trap technology in contrast to the more common technique of quadrupole detection. The advantages of the ion trap over the quadrupole are that it is possible to evaluate the chromatogram in both full scan mode and single ion monitoring. The ion trap is also more robust and less prone to pressure fluctuations when analysing

highly concentrated samples which would otherwise cause the pressure sensors of a quadrupole based system to switch the detector off and abort the analysis.

A common analysis program was developed to be applied to the analysis of all samples prepared and submitted for routine screening by GCMS. The Saturn 3 instrumental conditions are shown in Table 2.9.

Table 2.9 Saturn 3 GCMS instrumental conditions

Instrument	Varian Saturn 3 (Ion Trap)
Column	30 m × 0.32 mm DB5MS capillary column
Injector temperature	275 °C
Transfer line temperature	310 °C
Manifold temperature	170 °C
Column oven program	
Initial temperature	35 °C for 5 minutes
1st segment	5 °C/min to 200 °C
2nd segment	10 °C/min to 280 °C
3rd segment	15 °C/min to 310 °C
Final temperature	310 °C held for 5 minutes
Injection volume	3µl (fast injection mode 10 µl/min)
Ion Trap conditions	
Mass range	35–375 *m/z*
Background mass	34 *m/z*
Ion mode	Electron Impact (EI)
Filament Mul Delay	3.5 minutes

Water soluble polar organic compounds were determined by high performance liquid chromatography (HPLC) using Varian liquid chromatographs, models Vista 5500 and LC Star modular system in conjunction with a diode array (polychromator) detector. All separations were achieved using gradient elution with mobile phases based on the following systems: acetonitrile/water, with cetyltrimethylammonium bromide (CTAB) modifier and potassium dihydrogen orthophosphate as a buffer, acetonitrile/water plus acetic acid and methanol/water based eluents. A number of columns were used such as Zorbax RP8, Lichrosorb RP8, metsphere ODS; essentially all these columns are based on silica of varying surface properties and particle size. The HPLC methods and conditions, with specific column and mobile phase details, are listed in the following tables.

Table 2.10 Method 1.

Instrument	Varian Vista 5500
Detector	Varian 9065 polychromator (diode-array)
Column	200 × 4.4 mm Lichrosorb RP18 silica
Column temperature	35 °C
Injection volume	200 µl (Rheodyne loop injector)
Detector wavelength	254 nm
Eluents	
A: acetonitrile (far UV grade) + 0.025% w/v CTAB	
B: aqueous 0.01 M potassium dihydrogen orthophosphate + 0.025% w/v CTAB	
CTAB cetyltrimethylammonium bromide	

Gradient profile

Time	%A	%B	Flow rate (cm^3/min)
0	35	65	0.80
5	35	65	0.80
13	60	40	1.00
18	60	40	1.00
21	95	5	1.00
23	95	5	1.00
30	35	65	0.80

Table 2.11 Method 2.

Instrument	Varian LC Star
Detector	Varian 9065 polychromator (diode-array)
Column	200 × 4.4 mm Lichrosorb RP18 silica
Column temperature	35 °C
Injection volume	200 µl (Rheodyne loop injector)
Detector wavelength	254 nm
Eluents	
A: 90:10 acetonitrile (far UV grade) : UHQ water + 0.32 g ammonium acetate	
B: 10:90 acetonitrile (far UV grade) : UHQ water + 0.32 g ammonium acetate	

Gradient profile

Time	%A	%B	Flow rate (cm^3/min)
0	20	80	1.20
25	60	40	1.20
28	85	15	1.20
33	85	15	1.20
36	20	80	1.20
40	20	80	1.20

Table 2.12 Method 3.

Instrument	Varian LC Star
Detector	Varian 9065 polychromator (diode-array)
Column	200 × 4.4 mm Lichrosorb RP18 silica
Column temperature	35 °C
Injection volume	200 µl (Rheodyne loop injector)
Detector wavelength	254 nm
Eluents	
A:	90:10 acetonitrile (far UV grade) : UHQ water + 0.32 g ammonium acetate
B:	10:90 acetonitrile (far UV grade) : UHQ water + 0.32 g ammonium acetate

Gradient profile

Time	%A	%B	Flow rate (cm^3/min)
0	2	98	1.00
5	5	95	1.00
30	50	50	1.00
35	50	50	1.00
40	90	10	1.00
43	2	2	1.00
45	2	2	1.00

Table 2.13 Method 4.

Instrument:	Varian Vista 5500.
Detector:	Varian 9065 polychromator (diode-array)
Column:	260 × 4.6 mm Beckman Ultrasphere ODS
Column temperature	35 °C
Injection volume:	200 µl (Rheodyne loop injector)
Detector wavelength:	254 nm
Eluents:	
A:	acetonitrile (far UV grade) + 0.25% w/v CTAB
B:	aqueous 0.1 M potassium dihydrogen orthophosphate + 0.25% w/v CTAB
CTAB cetyl trimethyl ammonium bromide	

Gradient profile

Time	%A	%B	Flow rate (cm^3/min)
0	30	70	1.00
3	30	70	1.00
8	60	40	1.70
10	60	40	1.70
15	80	20	2.00
18	80	20	2.00
20	30	70	1.00
25	30	70	1.00

Table 2.14 Method 5.

Instrument	Varian Vista 5500.
Detector	Varian 9065 polychromator (diode-array)
Column	260 × 4.6 mm Beckman Ultrasphere ODS
Column temperature	35 °C
Injection volume	200 µl (Rheodyne loop injector)
Detector wavelength	254 nm
Eluents	
A: acetonitrile (far UV grade) + 0.25% w/v CTAB	
B: aqueous 0.1 M potassium dihydrogen orthophosphate + 0.25% w/v CTAB	
CTAB cetyl trimethyl ammonium bromide	

Gradient profile

Time	%A	%B	Flow rate (cm^3/min)
0	35	65	1.00
5	35	65	1.00
13	60	40	1.70
18	60	40	1.70
21	85	15	2.00
23	85	15	2.00
30	35	65	1.00
35	35	65	1.00

Table 2.15 Method 6.

Instrument	Varian LC Star
Detector	Varian 9065 polychromator (diode-array)
Column	200 × 4.4 mm Lichrosorb RP18 silica
Column temperature	35 °C
Injection volume	200 µl (Rheodyne loop injector)
Detector wavelength	254 nm
Eluents	
A: 90:10 acetonitrile (far UV grade) : UHQ water + 0.32 g ammonium acetate	
B: 10:90 acetonitrile (far UV grade) : UHQ water + 0.32 g ammonium acetate	

Gradient profile

Time	%A	%B	Flow rate (cm^3/min)
0	24	76	1.00
6	62	38	2.20
11	100	0	2.50
15	100	0	2.50
18	24	76	1.00
30	24	76	1.00

Table 2.16 Method 7.

Instrument	Varian Vista 5500
Detector	Varian 9065 polychromator (diode-array)
Column	260 × 4.6 mm Beckman Ultrasphere ODS
Column temperature	35 °C
Injection volume	200 µl (Rheodyne loop injector)
Detector wavelength	254 nm
Eluents	
A: acetonitrile (far UV grade) + 0.25% w/v CTAB	
B: aqueous 0.1 M potassium dihydrogen orthophosphate + 0.25% w/v CTAB	
CTAB cetyl trimethyl ammonium bromide	

Gradient profile

Time	%A	%B	Flow rate (cm^3/min)
0	35	65	0.80
5	35	65	0.80
13	60	40	1.70
18	60	40	1.70
21	85	15	2.00
23	85	15	2.00
30	35	65	0.80

Table 2.17 Method 8.

Instrument	Varian LC Star
Detector	Varian 9065 polychromator (diode-array)
Column	250 × 4.6 mm Lichrosorb RP8 silica
Column temperature	35 °C
Injection volume	200 µl (Rheodyne loop injector)
Detector wavelength	254 nm
Eluents	
A: 90:10 acetonitrile (far UV grade) : UHQ water + 0.32 g ammonium acetate	
B: 10:90 acetonitrile (far UV grade) : UHQ water + 0.32 g ammonium acetate	

Gradient profile

Time	%A	%B	Flow rate (cm^3/min)
0	10	90	1.00
15	100	0	1.20
22	100	0	1.50
25	10	90	1.50

2.3 Sample preparation techniques

Various procedures were applied to the preparation of samples prior to analysis, the objective of which was to both pre-concentrate and clean up the samples to remove any interfering components. These are described below.

2.3.1 Liquid-liquid extraction

The simplest technique for preparation of liquid samples is that of liquid-liquid extraction (LLE). In this technique, the solution containing analytes of interest is mixed with an immiscible solvent, in this case dichloromethane. The sample (typically 100 cm^3) is placed in a 250 cm^3 beaker and 50 cm^3 dichloromethane added along with a magnetic stirrer bar. The beaker is then placed on a stirrer plate and the contents stirred for 15 minutes to produce a well-mixed suspension of the dichloromethane in the aqueous layer. The sample aliquot and dichloromethane is then transferred to a separating funnel and the lower organic layer filtered through a filter paper containing anhydrous sodium sulphate; this removes any traces of water trapped with the solvent. The dried organic phase is then evaporated down to 1 cm^3 using a Zymark Turbovap Closed Cup concentrator at 45 °C. The concentrate is then transferred to a standard 2 cm^3 glass autosampler vial. The limitations of this procedure are that it only isolates non-polar and moderately polar organic compounds. Very polar compounds such as phenol, morpholine and certain benzothiazole derivatives, tend to be either poorly recovered or not extracted at all.

2.3.2 Solid phase extraction

To isolate the more polar compounds, the technique of solid phase extraction (SPE), was applied. It involves the use of disposable solid phase extraction cartridges (Figure 2.9, overleaf). These provide a rapid and cheap method of analyte isolation and purification.

The cartridges have to be conditioned prior to use in any analysis. The procedure is as follows. The cartridge is filled with UHQ water, which is slowly drawn through the sorbent bed using vacuum. This procedure is carried out twice and then repeated using two cartridge volumes of methanol. The sorbent bed is not allowed to dry out after the methanol treatment step. The cartridge is then washed with a further two aliquots of UHQ

water and finally the cartridge is filled with UHQ water. A sample line is attached to the SPE cartridge and the other end inserted into a prepared sample.

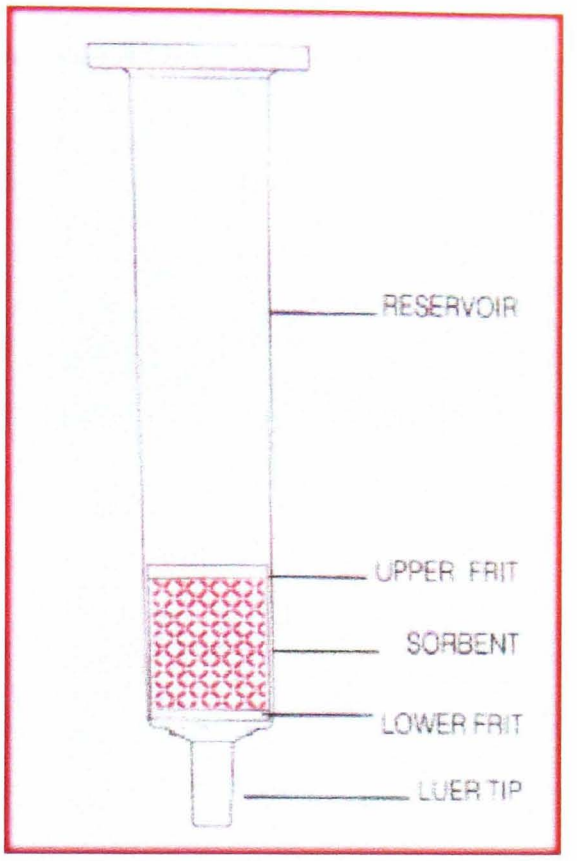


Figure 2.9 Typical solid phase extraction (SPE) cartridge.

The sample to be processed, typically a 500 cm^3 aliquot, is first filtered to remove any large insoluble particles which may block the sorbent bed during processing. The sample, is then treated with sodium chloride solution (10% w/v) and hydrochloric acid (1 cm^3 Analar grade) prior to passing through the SPE cartridge. The sample line is then inserted into the solution and vacuum applied to draw the water up the line and into the still wet cartridge. The vacuum is adjusted to maintain a steady flow of approximately 15 cm^3/min. At this flow rate it takes 30 minutes to process a 500 cm^3 sample and achieve a good recovery of analytes from the sample solution.

Solid phase extraction is a relatively new technique which provides a quick alternative to the more established liquid:liquid extraction. The approach to SPE is essentially the opposite of that used for adsorption chromatography. In the latter, the unwanted material is usually retained on the column while the analytes are eluted. In SPE, the analytes are retained on the sorbent in the cartridge as the sample solution is washed through. The analytes are then eluted at a secondary or tertiary stage using a different solvent.

Figure 2.10 shows a diagrammatic representation of how the isolation procedure is carried out; Figure 2.11 (overleaf) is a schematic of the system used to perform SPE extraction.

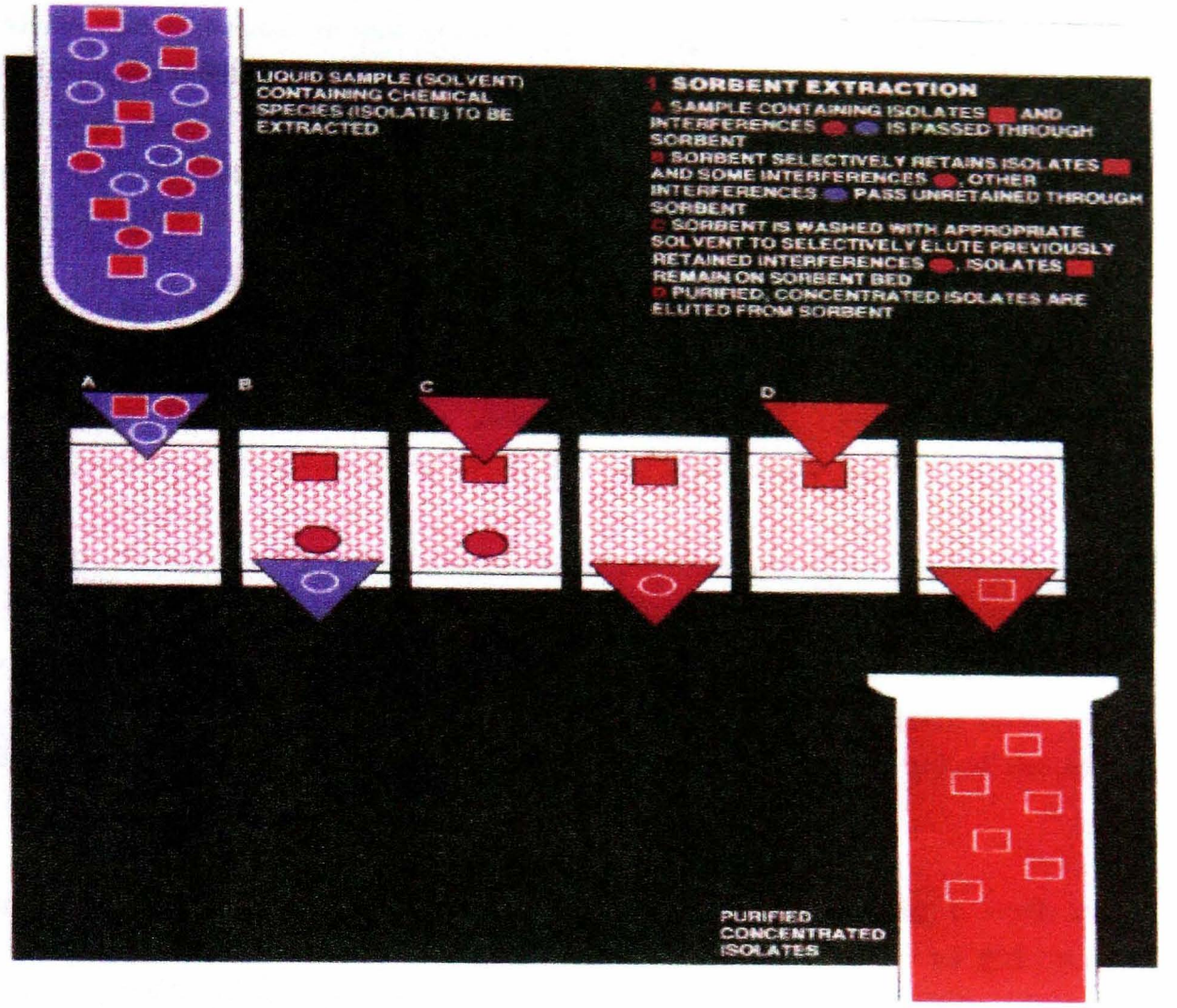


Figure 2.10 Schematic of SPE use. (Source: Varian sample preparation technical booklet; diagram courtesy of Varian UK Ltd.)

Two specific sorbents were used to clean up and isolate the various fractions of interest, Isolute™ C18 and Isolute™ ENV+ (Isolute is a trade name of IST the manufacturer of the SPE cartridges used). The cartridges were used in two modes: firstly, they were used separately for rapid screening analysis; secondly, they were used in a mixed mode (piggybacked), *i.e.*, the C18 cartridge was placed on top of the ENV+ cartridge so that it isolated unwanted less polar to non-polar components and allowed the more polar fractions to be retained on the ENV+ cartridge. Figure 2.12 (overleaf) shows a schematic of the retention and elution process on a ENV+ surface.

The solvent used to elute analytes determined the end analysis technique, *i.e.*, methanol or acetonitrile used primarily for HPLC analysis whereas acetonitrile was the solvent of choice for GCMS analysis.

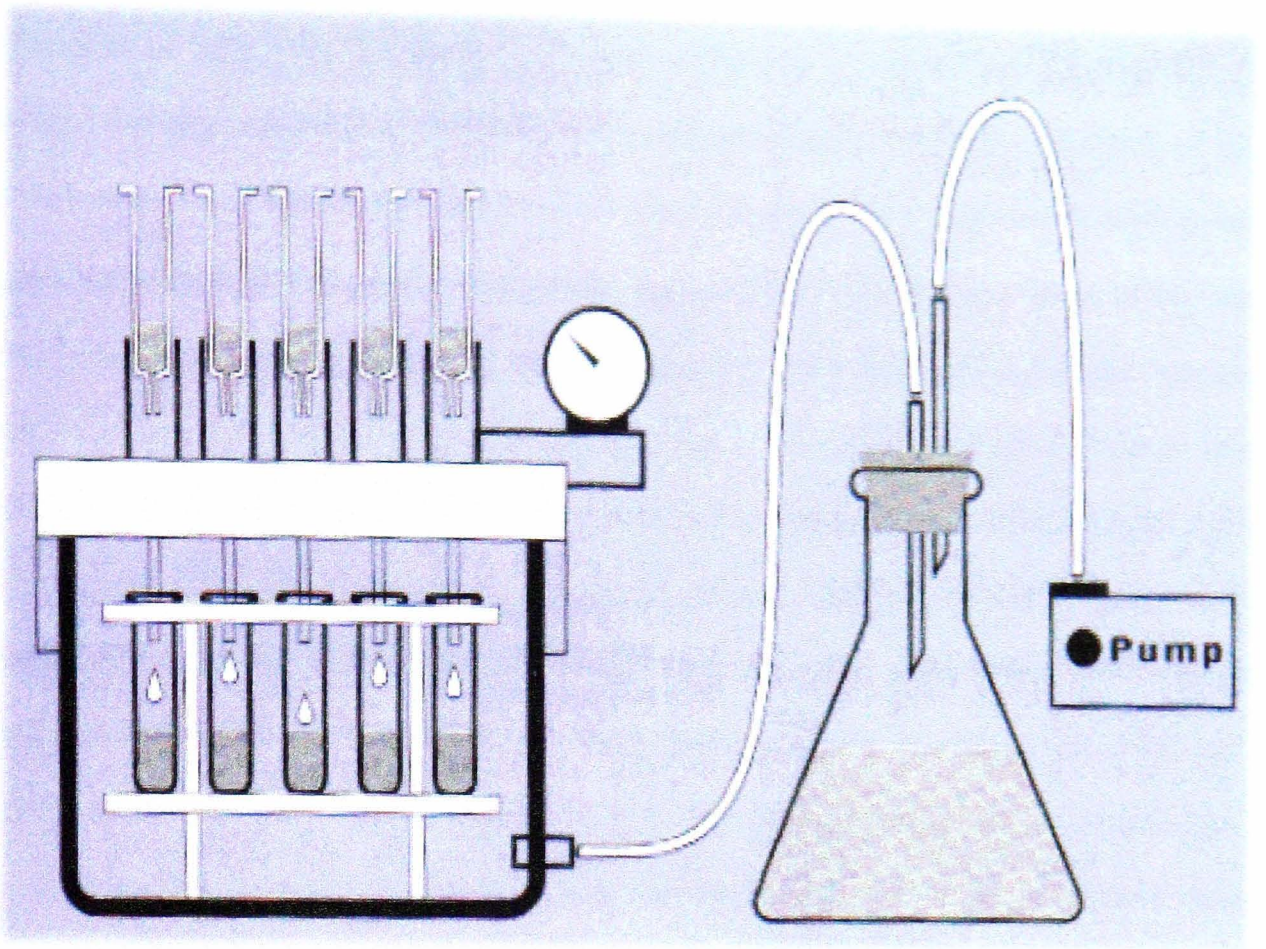


Figure 2.11 Diagram of apparatus used to carry out an SPE extraction. (Courtesy of Phenomenex UK Ltd.)

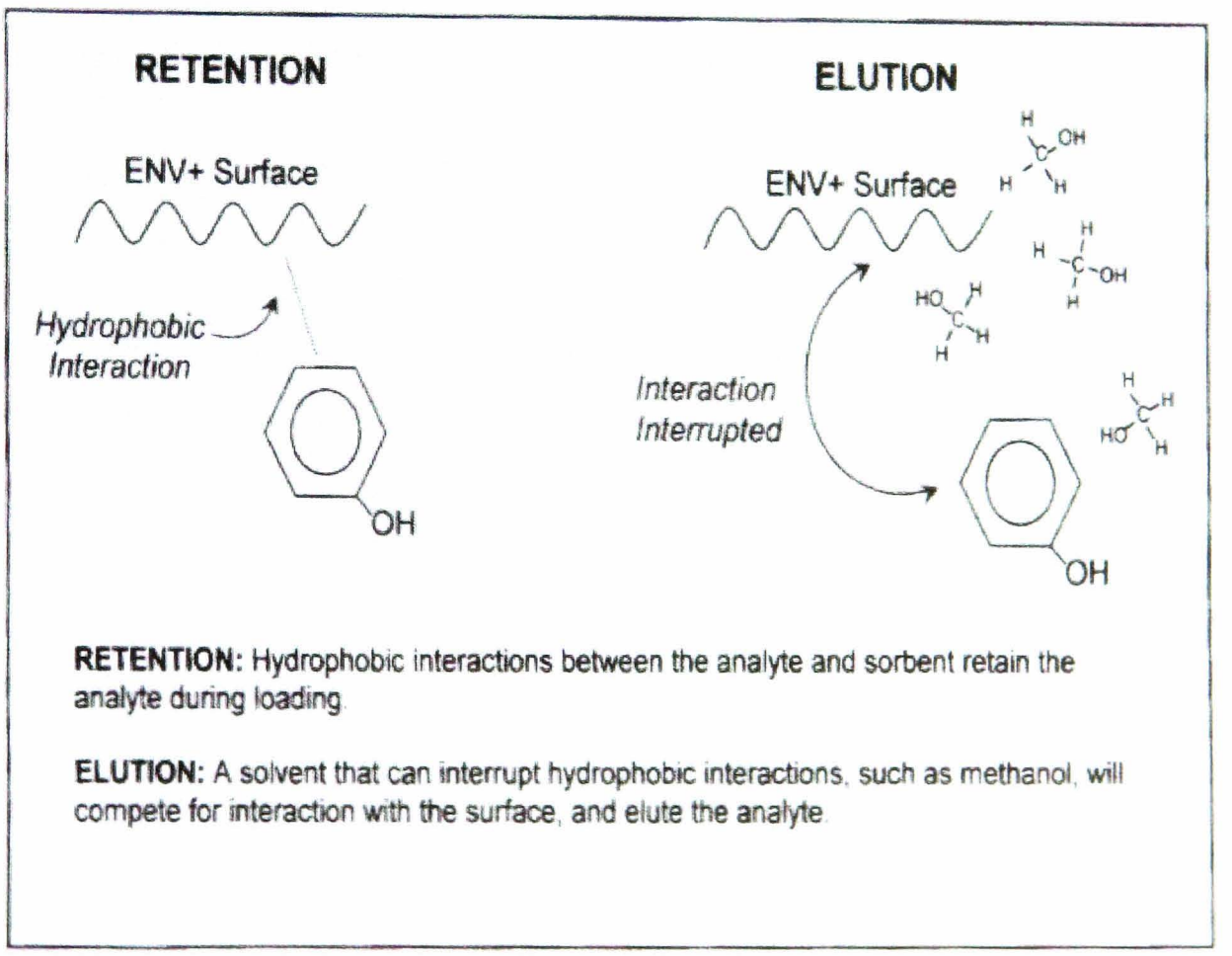


Figure 2.12 Retention process for ENV+ SPE cartridge. (Source: IST technical manual 'The Use of SPE Techniques'; diagram courtesy of IST Inc, USA.)

The benefits of SPE over the more traditional technique of liquid-liquid extraction (LLE) are many, the most significant being the low solvent usage to elute the analytes of interest after isolation. More selective isolations can be performed: in a mixture of both polar and non-polar analytes the use of two SPE cartridges allows selective isolation of the various species present in a single extraction step whereas the technique of LLE would necessitate the use of two separate extraction steps. Extraction of very polar compounds from water is difficult at best if not impossible, since generally a polar solvent would be most suited to the extraction of polar compounds. However, in the case of aqueous samples this is difficult due to the fact that most polar solvent are miscible with water. For example the extraction of phenolic compounds from water produces poor results unless chemical pre-treatment of the phenols is first carried out as in the method based on the standard method for phenol. In this method the phenol is first converted to the 4-aminoantipyrene complex. In this form the phenol is more readily extracted using a non-polar solvent such as chloroform. By contrast the extraction of phenol using the ENV+ SPE cartridge is a simple one step process involving no pre-treatment of the phenol and using only 2 cm^3 methanol to elute the phenol from the SPE cartridge (see Figure 2.12 which depicts the retention process using phenol as the example).

2.3.3 Validation of extraction procedures

In order to validate the extraction efficiency using both liquid:liquid extraction and SPE techniques a series of standard solutions were prepared containing known concentrations of the various analytes to be determined and the determinands isolated using the above techniques.

The isolates were analysed in the laboratories at Flexsys Ruabon and also by an external laboratory (Deejol Laboratories at Huntington Chester). The Deejol laboratory is the analytical section of the Welsh Water and provides the monitoring services to the Environment Agency. They have responsibility for daily monitoring of the River Dee and work in close association with the various industries along the banks of the River Dee.

The results obtained from Deejol laboratories were in good agreement with the results obtained by the Flexsys laboratory. A further analysis was carried out in which a second

set of standard solutions were prepared which were extracted in the two laboratories and the isolates obtained analysed by each laboratory. The isolates were then swapped between the laboratories and analysed a second time. The four sets of data were then compared and statistically evaluated to determine any significant differences between the laboratories.

The conclusion drawn from the work was that both laboratories were working in close agreement and that the proposed extraction techniques were applicable to the analysis of aqueous samples.

2.4 Experimental timeline for the biological reactors

The sequence of experiments was based on a 10–12 day metabolisation study followed by a period of washout between each experimental run (to equilibrate the biomass to the standard feed and/or the synthetic feed). In a small number of cases experiments were run back to back over a 20 day period. The washout time varied from between two weeks up to four weeks and was based on analysis of the discharge from the bioreactors for the absence of parent substrates and associated metabolites. The timeline sequence of events for the three bioreactors over the period of the research are shown in Appendix 2G–Appendix 2K.

Appendixes 2A–2F Full analytical data for all three bioreactors*

Appendix 2A Full analytical data for bioreactor R1 during baseline data acquisition

Time/days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Biological reactor analysis															
pH REACTOR R1	8.12	8.05	8.06	7.94	7.23	7.51	7.41	7.29	7.2	7.54	7.96	7.76	7.78	7.79	7.86
Temp Deg C R1	23.2	23.4	24.8	25.4	24.8	24	23.4	23	24.6	26.4	25.4	25	23.6	26	25
DISSOLVED OXYGEN R1	1.7	1.2	2.7	3.8	7.6	5.8	6.4	1.9	3.6	2.3	2.7	1.4	1.8	2.3	3.4
ALKALINITY R1	723	734	721	705	693	645	601	572	880	905	913	875	894	866	883
MLSS R1	12808	12228	12828	13040	12476	12605	12508	6284	9184	11552	8904	12646	12532	11196	9812
Effluent analysis															
COLOUR R1	0.84	0.88	1.02	1.06	1.24	1.22	1.22	1.2	1.18	1.02	1.13	1.09	0.96	0.98	0.73
AMMONIA R1	0.31	0.18	0.23	0.24	0.18	0.23	0.13	0.14	0.11	0.23	0.17	0.19	0.11	0.16	0.08
NITRATE R1	5.5	5.62	7.66	6.62	8.24	11.63	13.96	13.34	14.11	13.46	12.88	14.78	16.46	15.33	14.89
NITRITE R1	0.7	0.9	1.08	0.44	0.28	0.31	0.18	0.5	0.01	0.27	2.9	3	7.4	5.6	4.3
CHLORIDE R1	2361	2552	2638	2711	3037	3752	3876	3766	3874	3278	2838	3155	3029	2701	2738
SULPHATE R1	1726	1708	2052	1778	1821	1943	1969	2055	1919	2034	1785	2045	2177	2143	1785
TOC R1	40.5	46.2	52.8	80.4	73.9	59.4	64.3	68.2	66.1	50.7	54.3	48.4	51.6	49.5	64
ANILINE R1	1.28	0.32	0.81	0.73	0.65	0.43	0.92	0.97	0.75	0.65	0.76	0.65	0.47	0.76	0.58
BENZOTHIAZOLE R1	1.11	1.63	3.64	0.25	0.19	0.08	0.12	0.69	1.8	0.72	0.88	0.72	0.15	0.13	0.09
FLECTOL A R1	39.24	33.17	28.61	22.68	18.77	14.22	17.55	14.94	16.59	5.32	11.7	5.32	11.28	9.05	8.5
DMK R1	53.57	56.26	56.7	52.17	49.63	41.25	60.03	62.99	73.93	40.83	29.05	40.83	25.72	21.5	43.77

Time/days	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Biological reactor analysis															
pH REACTOR R1	7.84	7.91	7.95	7.85	7.99	7.93	7.94	7.95	7.95	7.89	8.03	7.66	7.99	7.51	8.06
Temp Deg C R1	24.4	24.4	23.4	24.1	23.6	25.4	23.7	24.8	23	23.8	23.9	24.3	24.4	23.3	24.5
DISSOLVED OXYGEN R1	3.8	3.1	4.1	5.2	4.6	4	2.8	2.1	1.6	1.1	0.7	0.3	1.1	2	2.2
ALKALINITY R1	916	925	960	933	904	882	856	850	826	800	792	747	756	779	736
MLSS R1	10804	10956	11068	11252	13048	11512	12436	12312	12576						
Effluent analysis															
COLOUR R1	0.73	0.72	0.41	0.61	0.54	0.51	0.49	0.43	0.38	0.39	0.56	0.35	0.39	0.23	0.24
AMMONIA R1	0.09	0.08	0.06	0.11	0.1	0.32	0.11	0.29	0.31	0.42	0.56	0.22	0.27	0.15	0.19
NITRATE R1	15.24	14.42	8.64	5.22	4.66	4.24	3.82	2.98	2.28	1.82	1.5	2.16	2.6	4.96	5.16
NITRITE R1	3.7	2.9	2.4	1.3	1.8	1.6	1.6	1.1	1.1	1.3	1.8	1.2	0.95	0.82	0.79
CHLORIDE R1	2813	2882	2738	2613	3062	2991	2788	2777	2889	3142	3193	2788	3248	3175	2818
SULPHATE R1	1863	1756	1635	1529	2135	2095	1939	2117	2239	1987	1754	1419	1559	1411	1371
TOC R1	79.3	81.2	88.4	96.5	91.2	79.6	65	71	89.2	113	123.6	130.1	147.4	101.2	93.4
ANILINE R1	0.22	0.18	0.11	0.23	0.26	0.37	0.64	0.53	0.37	1.14	2.79	1.12	0.41	0.41	0.1
BENZOTHIAZOLE R1	0.1	0.18	0.35	0.11	0.34	0.45	1.34	0.16	0.17	3.13	7.32	9.76	6.62	4.44	1.2
FLECTOL A R1	6.33	4.71	4.55	3.68	5.47	8.21	13.6	21.27	28.35	33.48	31.27	28.69	24.23	28.44	23.64
DMK R1	36	28.4	56.03	40.73	42.32	50.78	59.92	33.47	44.34	51.71	50.33	47.59	30.83	40.9	46.28

*All analytical values for the various determinands are in mg/l.

Time/days	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Biological reactor analysis															
pH REACTOR R1	7.75	7.87	7.96	8.02	8	8.1	8.05	8.02	7.95	7.98	7.81	7.9	7.92	7.81	7.88
Temp Deg C R1	26.7	26.4	25.4	23.3	24	23	23	23	23.1	24.7	25.5	24.7	23.3	23.4	22.9
DISSOLVED OXYGEN R1	3.3	2.8	2.4	2.5	2.4	2.1	2.7	2.3	2.9	1.9	1.3	2.1	2.5	2.2	2.6
ALKALINITY R1	748	785	792	827	817	837	875	883	900	635	688	724	736	709	732
MLSS R1															
Effluent analysis															
COLOUR R1	0.38	0.69	0.81	0.9	0.86	0.93	1.05	1.13	0.97	0.83	1.07	1.11	1.12	1.05	0.96
AMMONIA R1	0.18	0.12	0.14	0.19	0.23	0.12	0.11	0.1	0.22	0.13	0.11	0.09	0.14	0.19	0.11
NITRATE R1	7.18	9.11	8.41	7.24	9.18	10.36	8.32	7.43	6.95	5.48	4.64	3.42	4.22	2.94	3.92
NITRITE R1	0.45	4.13	4.7	3.21	2.12	0.93	0.3	0.13	0.1	0.14	0.09	0.05	0.01	0.02	0.08
CHLORIDE R1	2906	2760	2636	3041	3160	3310	3227	3115	3036	2919	3026	3112	3235	3433	3379
SULPHATE R1	1451	1800	1696	1710	1863	1912	1856	1792	1726	1819	1762	1810	1823	1796	1701
TOC R1	91.4	61.3	46.2	53.2	71.2	79.3	63.7	51.4	43.2	35.7	44.2	59.3	63.4	71.1	63.4
ANILINE R1	0.06	0.03	0.11	0.13	0.02	0.21	0.2	0.29	0.31	0.27	0.2	0.32	0.06	0.07	0.1
BENZOTHIAZOLE R1	0.86	0.21	0.17	0.09	0.31	0.71	0.61	0.28	0.16	1.11	2.36	1.17	0.6	0.11	0.09
FLECTOL A R1	18.76	6.79	4.63	2.22	7.11	15.2	21.82	17.63	29	19.51	22.34	25.41	27.24	21.27	17.9
DMK R1	36.24	17.11	22.61	42.24	63.93	41.27	21.92	24.73	49.37	31.11	35.17	26.5	41.11	36.75	23.92

Time/days	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Biological reactor analysis															
pH REACTOR R1	7.72	7.79	8	8.05	7.92	8.03	7.93	7.81	7.92	7.86	7.93	7.99	8.02	8.11	8.05
Temp Deg C R1	22.7	23	22.8	21.9	22.5	22.6	23.1	23.4	22.4	22.6	22.4	21.9	21.5	22	22.4
DISSOLVED OXYGEN R1	2.1	1.5	1.9	2.4	2.7	2.3	2.7	2.3	2.8	3.1	2.7	2.4	2.7	2.2	2.5
ALKALINITY R1	610	623	640	662	671	683	676	647	668	701	723	730	705	693	673
MLSS R1															
Effluent analysis															
COLOUR R1	0.72	0.75	0.83	0.91	1.05	0.86	0.91	0.9	0.83	0.79	0.66	0.77	0.91	0.72	0.83
AMMONIA R1	0.09	0.11	0.26	0.21	0.32	0.26	0.24	0.19	0.16	0.21	0.13	0.15	0.13	0.11	0.09
NITRATE R1	5.14	7.28	6.14	4.24	4.48	4.62	3.22	3.94	4.12	4.24	3.7	3.26	3.14	3.38	3.48
NITRITE R1	0.11	0.13	0.1	0.09	0.06	0.03	0.05	0.2	0.14	0.1	0.13	0.11	0.14	0.11	0.09
CHLORIDE R1	3055	2966	3019	3127	3278	3210	3063	2841	2671	2759	2905	3015	3145	3215	3063
SULPHATE R1	1896	1926	1790	1900	1925	1853	1927	2115	2137	2003	1973	2076	2146	1953	1984
TOC R1	52.2	48.3	43.9	37.2	43.6	55.5	47.2	59.1	62.3	52.4	58.3	53.1	50.1	48.2	41.7
ANILINE R1	0.11	0.08	0.11	0.09	0.22	0.14	0.2	0.18	0.11	0.14	0.21	0.14	0.11	0.06	0.11
BENZOTHIAZOLE R1	0.21	0.32	0.19	0.09	0.35	0.39	0.21	0.24	0.29	0.33	0.35	0.32	0.24	0.36	0.21
FLECTOL A R1	21.1	2	3.09	0.51	2.67	3.91	16.22	18.95	21.17	18.32	16.45	9.87	4.35	2.11	3.54
DMK R1	51.3	62.3	67.91	72.14	52	61.71	45.38	48.64	45.69	48.33	51.32	56.66	48.75	43.25	46.51

Time/days	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
Biological reactor analysis															
pH REACTOR R1	7.96	7.91	7.88	7.85	7.75	7.87	7.93	7.94	7.81	7.76	7.84	7.91	7.99	7.86	7.84
Temp Deg C R1	21.6	22	22.3	22.6	21.8	21.4	21	21.1	21.5	21.8	22.3	22.5	22.8	22.9	22.4
DISSOLVED OXYGEN R1	2.1	2.7	2.3	2	2.4	2	1.8	2.1	2.4	2.6	2.1	2	2.2	2.4	2.8
ALKALINITY R1	660	611	595	604	586	556	529	531	520	502	493	478	423	436	416
MLSS R1															
Effluent analysis															
COLOUR R1															
AMMONIA R1	0.14	0.18	0.24	0.31	0.22	0.2	0.17	0.2	0.24	0.26	0.31	0.33	0.24	0.25	0.21
NITRATE R1															
NITRITE R1															
CHLORIDE R1															
SULPHATE R1															
TOC R1	48.6	41.3	42.2	39.4	37.6	34.6	38.8	37.1	34.8	38.9	34.8	36.7	37.2	35.9	37.4
ANILINE R1															
BENZOTHIAZOLE R1															
FLECTOL A R1															
DMK R1															

Time/days	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Biological reactor analysis															
pH REACTOR R1	7.88	7.93	8.01	8.2	8.24	8.11	8.04	8.01	7.98	7.95	7.9	7.94	7.86	7.78	7.72
Temp Deg C R1	22.5	22	21.8	21.6	21.7	22.3	21.6	21.3	21	21.6	22	22.3	22.8	22.4	22.1
DISSOLVED OXYGEN R1	2.3	2	2.4	2.1	1.8	1.6	1.9	2.2	2.4	2.3	2.1	2.3	2.7	2.2	2.2
ALKALINITY R1	403	417	468	473	489	468	441	418	429	439	451	428	436	449	451
MLSS R1															
Effluent analysis															
COLOUR R1															
AMMONIA R1	0.19	0.14	0.21	0.26	0.3	0.24	0.23	0.27	0.21	0.25	0.26	0.27	0.24	0.21	0.19
NITRATE R1															
NITRITE R1															
CHLORIDE R1															
SULPHATE R1															
TOC R1	36	38.2	36.9	35.7	37.2	38.3	35.7	36.8	39.1	37.2	37.9	36.1	39.1	37.3	38.4
ANILINE R1															
BENZOTHIAZOLE R1															
FLECTOL A R1															
DMK R1															

Time/days	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
Biological reactor analysis															
pH REACTOR R1	7.58	7.68	7.77	7.71	7.8	7.84	7.89	7.76	7.79	7.85	7.89	7.95	7.97	7.86	7.81
Temp Deg C R1	22.5	22.9	22.6	22.4	22.7	22.4	22.6	22.7	22.1	21.8	21.6	21.7	21.9	21.6	22
DISSOLVED OXYGEN R1	2.6	2.1	2.4	2.1	2.3	2.5	2.1	1.8	2.1	2.5	2.2	2.4	2.4	2.1	2.6
ALKALINITY R1	463	450	442	462	473	481	505	517	502	524	511	531	519	500	515
MLSS R1															
Effluent analysis															
COLOUR R1															
AMMONIA R1	0.22	0.27	0.33	0.36	0.41	0.36	0.31	0.34	0.28	0.24	0.29	0.34	0.33	0.28	0.31
NITRATE R1															
NITRITE R1															
CHLORIDE R1															
SULPHATE R1															
TOC R1	37.2	36.7	35.1	37	37.9	36.2	36.8	37.1	35.8	34.9	36.2	36.9	38.4	39.1	37.3
ANILINE R1															
BENZOTHIAZOLE R1															
FLECTOL A R1															
DMK R1															

Time/days	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Biological reactor analysis															
pH REACTOR R1	7.77	7.72	7.85	7.94	7.99	8	7.98	7.86	7.88	7.95	7.81	7.88	7.78	7.86	7.92
Temp Deg C R1	22.3	22.6	22.7	22.4	22.6	22.1	21.8	21.6	21.8	22	22.3	22.7	22.4	22.7	22.3
DISSOLVED OXYGEN R1	2.3	2	2.1	1.9	2.2	2.4	2.1	2.3	2.3	2.1	2.5	2.2	2.4	2	2.3
ALKALINITY R1	521	503	511	500	517	526	537	520	509	511	521	508	517	500	516
MLSS R1															
Effluent analysis															
COLOUR R1															
AMMONIA R1	0.37	0.45	0.41	0.38	0.33	0.37	0.41	0.44	0.4	0.38	0.34	0.3	0.31	0.33	0.29
NITRATE R1															
NITRITE R1															
CHLORIDE R1															
SULPHATE R1															
TOC R1	38.1	36.7	35.7	36.8	37.8	35.4	35.8	36.9	36.6	36.1	37.5	37.9	36.4	37.2	
ANILINE R1															
BENZOTHIAZOLE R1															
FLECTOL A R1															
DMK R1															

Appendix 2B Full analytical data for bioreactor R2 during baseline data acquisition

Time/days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Biological reactor analysis															
pH REACTOR R2	8.11	8.05	8.05	7.96	7.68	7.49	7.68	7.21	7.3	7.63	7.8	7.84	7.85	7.82	7.92
Temp Deg C R2	23.2	24.5	24.8	25.4	24.8	24.1	23.4	23	24.8	26.4	25.2	25	23.4	25.6	24.8
DISSOLVED OXYGEN R2	1.9	1	2.2	4	7.1	5.9	6.8	1.2	3.2	2.5	3	2.1	2	2.1	2.7
ALKALINITY R2	741	753	736	711	687	657	616	566	900	918	902	894	912	900	891
MLSS R2	11868	12052	12472	12616	12204	11375	8760	9860	8140	11212	12892	14544	13720	11548	9800
Effluent analysis															
COLOUR R2	0.81	0.88	0.98	1.02	1.28	1.23	1.2	1.2	1.24	0.88	0.83	0.88	0.8	0.78	0.56
AMMONIA R2	0.21	0.18	0.16	0.14	0.12	0.12	0.08	0.1	0.11	0.3	0.24	0.08	0.09	0.12	0.09
NITRATE R2	4.54	5.62	6.22	6.68	7.86	8.91	12.44	12.3	13.62	9.46	12.14	11.76	13.68	14.28	15.46
NITRITE R2	1.1	0.9	1.2	0.19	0.13	0.36	0.27	0.26	0.01	0.89	3.5	2.6	6.2	4.6	4
CHLORIDE R2	2367	2552	2664	2744	2948	3648	3802	3775	3643	3390	3075	3135	3063	2776	2794
SULPHATE R2	1724	1708	2123	1809	1768	1901	1942	2007	1970	2029	1921	1998	2196	2166	1847
TOC R2	38.9	47.4	52.1	63.8	71.2	59.1	65.3	62.7	64.1	51.2	55	51.4	52.3	59.1	61.2
ANILINE R2	1.02	0.32	0.49	0.93	1.11	1.47	1.21	1.36	1.08	0.36	0.43	0.36	0.4	0.56	0.63
BENZOTHIAZOLE R2	1.14	1.63	2.68	0.31	0.21	0.15	0.14	0.88	1.54	0.56	0.51	0.83	0.77	0.24	0.3
FLECTOL A R2	34.58	25.09	23.47	13.34	18.64	11.17	14.3	11.64	12.71	5.45	15.68	12.81	13.03	12.27	22.06
DMK R2	48.27	56.26	63.65	70.49	56.34	42.36	72.18	61.57	71.39	51.95	29.58	30.54	27.48	26.11	43.05

Time/days	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Biological reactor analysis															
pH REACTOR R2	7.94	7.94	7.95	7.95	8.06	8.01	8.06	8.01	7.99	8.01	7.97	7.9	7.85	7.64	8.01
Temp Deg C R2	24.4	24.4	23.4	24	23.6	25.4	23.6	24.6	23	23.7	23.7	24.3	24.2	23.1	24.4
DISSOLVED OXYGEN R2	3.2	3.9	4.5	5.1	4.2	3.7	2.9	1.8	1.3	0.9	0.5	0	1.3	2.4	2.7
ALKALINITY R2	931	911	948	921	909	879	863	855	832	811	798	752	735	764	721
MLSS R2	10932	11005	9936	12856	9256	11356	12960	12332	12988						
Effluent analysis															
COLOUR R2	0.44	0.43	0.41	0.42	0.41	0.4	0.41	0.39	0.35	0.29	0.32	0.35	0.37	0.33	0.31
AMMONIA R2	0.1	0.07	0.06	0.06	0.08	0.19	0.1	0.39	0.27	0.56	0.45	0.31	0.28	0.14	0.11
NITRATE R2	15.87	16.21	8.68	4.44	3.52	3.36	3.28	2.44	2.54	1.66	1.88	2.37	2.82	5.11	6.96
NITRITE R2	3.4	2.8	2.1	1.5	1.5	1.6	1.7	1.1	1.3	1.5	2.1	1.3	0.87	0.91	0.71
CHLORIDE R2	2836	2900	2843	2748	3058	3017	2930	2797	2863	3159	3215	3358	3481	3421	3393
SULPHATE R2	1717	1657	1683	1599	2076	2076	2028	2123	2181	1979	1761	1759	1667	1586	1702
TOC R2	76.8	85.8	91.3	94.7	89.2	81	71.2	69.3	82	110	123.3	129.7	137.2	100.6	98.7
ANILINE R2	0.42	0.31	0.22	0.17	0.22	0.29	0.34	0.48	0.51	1.34	2.54	1.63	1.11	0.87	0.27
BENZOTHIAZOLE R2	0.05	0.11	0.28	0.09	0.22	0.37	1.65	0.78	0.38	2.12	3.87	5.48	4.89	3.55	1.82
FLECTOL A R2	15.35	12.86	11.76	5.33	11.3	19.45	31.19	42.23	37.1	38.86	24.23	28.67	21.33	27.82	24.12
DMK R2	25.73	26.61	44.54	44.68	51.23	59.36	68.94	54.62	52.85	53.95	54.48	53.17	35.68	44.28	49.77

Time/days	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Biological reactor analysis															
pH REACTOR R2	7.84	7.96	7.91	7.86	7.84	7.75	7.95	8	8	8.01	7.96	7.91	7.93	7.89	7.86
Temp Deg C R2	26.8	26.2	25.3	23	23.7	23	22.8	22.9	23.4	24.5	25.3	24.6	23.1	23.3	22.6
DISSOLVED OXYGEN R2	3.1	2.6	2.2	2.6	2.5	2.3	2.7	2.4	2.9	2.2	2	1.8	2.3	2	2.4
ALKALINITY R2	752	771	801	833	821	842	877	889	895	651	674	705	721	718	741
MLSS R2															
Effluent analysis															
COLOUR R2	0.31	0.61	0.79	0.96	0.91	0.87	0.92	1.1	0.89	0.86	0.98	1	1.16	1.12	1
AMMONIA R2	0.19	0.14	0.17	0.13	0.21	0.33	0.23	0.16	0.13	0.09	0.06	0.08	0.16	0.19	0.13
NITRATE R2	7.5	10.24	10.51	9.78	8.66	8.46	8.81	7.92	5.72	4.64	4.14	3.32	3.14	2.84	3.16
NITRITE R2	0.39	3.61	3.71	3.39	2.97	1.65	1.1	0.96	0.82	0.67	0.42	0.27	0.13	0.15	0.09
CHLORIDE R2	3873	2685	2717	2963	3019	3200	3293	3116	3056	3005	2990	3015	3163	3227	3416
SULPHATE R2	1982	1856	1710	1786	1900	1956	1823	1787	1823	1768	1820	1843	1861	1800	1797
TOC R2	92.5	59.3	46.7	56.4	66.8	76.3	64.5	54.1	50.4	41.8	46.3	58.4	61.4	69.3	61.2
ANILINE R2	0.07	0.04	0.08	0.1	0.03	0.17	0.16	0.2	0.23	0.16	0.23	0.21	0.06	0.03	0.09
BENZOTHIAZOLE R2	1.11	0.16	0.21	0.16	0.23	0.63	0.52	0.36	0.09	0.07	1.29	2.26	0.91	0.14	0.08
FLECTOL A R2	15.65	5.81	5	4.17	6.96	14.96	23.67	19.21	19	18.76	21.6	24.88	29.47	22.27	18.23
DMK R2	29.64	16.11	21.36	39.98	56.31	50.11	31.23	27.61	37.86	29.71	32.92	29.53	43.21	39.33	30.6

Time/days	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Biological reactor analysis															
pH REACTOR R2	7.82	7.85	7.97	7.88	7.83	7.96	8.02	7.97	8	7.96	8.01	8.05	8.13	8.04	8
Temp Deg C R2	22.7	22.9	22.6	21.8	22.5	22.4	22.9	23.1	22.7	22.9	23.1	22.7	22.2	21.9	21.6
DISSOLVED OXYGEN R2	1.9	1.7	2	2.2	2.5	2.1	2.5	2.1	2.6	2.9	2.5	2.1	2.5	2	2.4
ALKALINITY R2	600	617	636	658	679	694	681	658	674	693	718	741	711	700	681
MLSS R2															
Effluent analysis															
COLOUR R2	0.89	0.81	0.76	0.81	0.96	0.9	0.96	0.87	0.8	0.83	0.71	0.8	0.86	0.74	0.8
AMMONIA R2	0.12	0.04	0.09	0.11	0.18	0.21	0.32	0.27	0.21	0.17	0.18	0.13	0.16	0.12	0.1
NITRATE R2	3.52	3.96	5.24	5.66	5.3	4.34	3.66	3.78	3.92	4.14	3.86	3.58	3.28	3.14	3.28
NITRITE R2	0.07	0.1	0.08	0.03	0.05	0.06	0.09	0.11	0.18	0.13	0.09	0.11	0.12	0.09	0.07
CHLORIDE R2	3356	2995	2817	3092	3305	3211	3087	2915	2887	2731	2849	2985	3106	3233	3127
SULPHATE R2	1762	1873	1801	1888	1936	1903	1947	2090	2121	2095	2003	2111	2156	2003	2088
TOC R2	55.6	51.2	45.7	33.6	39.6	47.2	42.6	51.1	61.4	59.3	63.1	56.4	53.2	50.1	46.7
ANILINE R2	0.13	0.09	0.14	0.11	0.18	0.16	0.23	0.22	0.19	0.11	0.17	0.2	0.15	0.1	0.07
BENZOTHIAZOLE R2	0.13	0.22	0.25	0.12	0.26	0.34	0.22	0.27	0.3	0.36	0.28	0.21	0.26	0.31	0.33
FLECTOL A R2	23.62	17	6.11	2.15	3.11	4.62	12.98	17.66	23.62	17.66	12.34	8.51	3.69	2.17	3.11
DMK R2	49.27	58.96	61.28	73.21	63.37	59.09	52.17	48.72	42.16	46.36	51.14	49.57	46.33	44.21	42.98

Time/days	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
Biological reactor analysis															
pH REACTOR R2	7.92	7.87	7.78	7.73	7.77	7.91	7.95	7.91	7.9	7.84	7.88	7.91	7.84	7.81	7.88
Temp Deg C R2	21.4	21.7	22.1	22.7	22.2	21.7	21.3	21.1	21.3	21.6	22.1	22.6	22.4	23	22.8
DISSOLVED OXYGEN R2	2.2	2.8	2.4	2.2	2.5	2.1	2.2	1.9	2.3	2.8	2.2	2.3	2.5	2.1	2.5
ALKALINITY R2	657	629	601	613	598	563	541	529	511	500	502	493	445	456	429
MLSS R2															
Effluent analysis															
COLOUR R2															
AMMONIA R2	0.32	0.14	0.17	0.23	0.25	0.21	0.2	0.23	0.21	0.24	0.27	0.25	0.22	0.27	0.23
NITRATE R2															
NITRITE R2															
CHLORIDE R2															
SULPHATE R2															
TOC R2	44.4	40.9	43.3	40.4	37.8	36.6	38.4	36.6	34.8	37.6	36.4	35.7	37	36.4	38
ANILINE R2															
BENZOTHIAZOLE R2															
FLECTOL A R2															
DMK R2															

Time/days	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Biological reactor analysis															
pH REACTOR R2	7.92	7.99	8.06	8.14	8.22	8.23	8.17	8.08	8	7.89	7.88	7.79	7.83	7.88	7.74
Temp Deg C R2	22.7	22.4	21.9	21.4	21.6	21.9	21.3	21	21.1	21.5	21.9	22.3	22.6	22.9	22.5
DISSOLVED OXYGEN R2	2.1	1.9	2.2	1.9	1.7	2	2.2	2.4	2.3	2.5	2.8	2.6	2.3	2	2.4
ALKALINITY R2	411	423	447	461	480	471	456	431	440	453	460	447	459	463	450
MLSS R2															
Effluent analysis															
COLOUR R2															
AMMONIA R2	0.2	0.2	0.22	0.22	0.24	0.28	0.31	0.26	0.24	0.22	0.21	0.19	0.24	0.26	0.31
NITRATE R2															
NITRITE R2															
CHLORIDE R2															
SULPHATE R2															
TOC R2	37.1	39.2	37.8	36.3	35.2	37.1	36.4	36.9	38.2	39.4	38	37.2	36.4	39.7	36.8
ANILINE R2															
BENZOTHIAZOLE R2															
FLECTOL A R2															
DMK R2															

Time/days	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
Biological reactor analysis															
pH REACTOR R2	7.5	7.38	7.58	7.69	7.83	7.9	7.92	7.83	7.81	7.99	7.93	7.82	7.69	7.73	7.8
Temp Deg C R2	22.4	22.6	22.6	22.3	22.5	22.3	22.5	22.4	22.7	22.2	22	21.8	21.6	21.7	21.6
DISSOLVED OXYGEN R2	2.7	2.5	2.3	2.4	2.3	2.6	2.3	2	2.1	2.4	2.6	2.3	2.1	2	2.4
ALKALINITY R2	459	447	438	452	481	498	509	523	509	517	503	522	501	496	508
MLSS R2															
Effluent analysis															
COLOUR R2															
AMMONIA R2	0.42	0.47	0.39	0.34	0.33	0.27	0.25	0.26	0.27	0.26	0.3	0.31	0.37	0.32	0.27
NITRATE R2															
NITRITE R2															
CHLORIDE R2															
SULPHATE R2															
TOC R2	37.5	37.9	36.1	36.9	38.2	37.1	36.2	38	37.4	36.5	34.8	36	38.2	37.8	36.7
ANILINE R2															
BENZOTHIAZOLE R2															
FLECTOL A R2															
DMK R2															

Time/days	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Biological reactor analysis															
pH REACTOR R2	7.88	7.95	7.94	8.02	8.08	7.98	7.93	7.97	7.82	7.88	7.94	7.97	7.87	7.79	7.86
Temp Deg C R2	21.9	22.3	22.5	22.1	22.4	22.7	22.2	21.9	21.6	21.8	22	22.4	22.1	22.3	22.5
DISSOLVED OXYGEN R2	2.6	2.1	2.4	2.2	2.3	2	2.2	2.5	2.1	1.8	2	2.3	2.1	2.2	2.5
ALKALINITY R2	530	517	520	511	503	519	528	512	500	518	527	519	526	511	523
MLSS R2															
Effluent analysis															
COLOUR R2															
AMMONIA R2	0.33	0.33	0.42	0.41	0.38	0.4	0.42	0.48	0.41	0.4	0.35	0.33	0.34	0.36	0.31
NITRATE R2															
NITRITE R2															
CHLORIDE R2															
SULPHATE R2															
TOC R2	36.9	37.5	36.1	35.4	36.4	36	36.1	36.4	37	37.2	38	38.3	37.5	36.6	37.2
ANILINE R2															
BENZOTHIAZOLE R2															
FLECTOL A R2															
DMK R2															

Appendix 2C Full analytical data for bioreactor R3 during baseline data acquisition

Time/days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Biological reactor analysis															
pH REACTOR R3	8.18	8.15	8.23	8.15	7.82	7.62	7.36	7.31	7.33	7.67	7.86	7.88	7.91	7.87	8.03
Temp Deg C R3	24	25.1	25.4	26	25	24.6	24	23.4	25	27	25.2	25.6	24.2	26.2	25.4
DISSOLVED OXYGEN R3	1.6	1.3	2.9	4.2	6.8	6.1	5.3	2.3	3.7	2.9	2.6	1.9	2.3	2.5	2.8
ALKALINITY R3	736	741	738	721	700	685	636	603	860	902	911	895	936	903	875
MLSS R3	11352	11016	11132	10628	13272	12635	9220	9888	6496	12084	11312	12480	11780	10985	9615
Effluent analysis															
COLOUR R3	0.76	0.86	0.88	0.88	1	1.003	1.04	1.04	0.92	0.86	0.88	0.9	0.86	0.84	0.58
AMMONIA R3	0.36	0.15	0.12	0.1	0.08	0.08	0.07	0.07	0.06	0.15	0.2	0.07	0.06	0.21	0.18
NITRATE R3	3.86	4.72	5.7	5.84	6.2	10.76	11.76	11.42	12.76	12.54	11.94	11.7	13.38	13.98	15.27
NITRITE R3	1	1.2	1.25	0.32	0.34	0.14	0.12	0.39	0.01	0.69	3.4	2.7	6.4	7.3	5.1
CHLORIDE R3	2332	2541	2720	2805	3009	3757	3783	3955	3857	3451	2939	2986	2888	2739	2712
SULPHATE R3	1712	1692	1983	1848	1807	1945	1918	2000	2018	2069	1827	1877	2038	2114	1770
TOC R3	42.3	50.1	58.3	58.4	65.7	63.8	62.4	65.4	67.8	62.8	57.6	53.9	50.3	53.4	65.7
ANILINE R3	0.85	0.46	0.33	0.4	0.67	1.13	1.27	1.85	0.55	0.87	0.59	0.87	0.4	0.49	0.68
BENZOTHIAZOLE R3	0.087	1.48	1.99	0.11	0.13	0.06	0.08	0.57	1.11	0.76	1.07	1.24	0.96	0.48	0.24
FLECTOL A R3	34.58	25.09	23.47	13.34	18.64	11.17	14.3	11.64	12.71	5.45	15.68	12.81	13.03	12.27	22.06
DMK R3	31.63	58.14	61.79	76.74	48.5	31.16	51.51	65.59	29.02	45.3	28.67	45.3	27.48	18.83	39.55

Time/days	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Biological reactor analysis															
pH REACTOR R3	7.97	7.97	7.95	7.97	8.19	8.06	8.08	7.93	8.01	8.03	7.98	7.93	7.9	7.71	7.97
Temp Deg C R3	25	24.8	23.8	24.6	24.3	25.9	24.1	24.2	23.6	24	24.1	24.7	24.8	23.4	25
DISSOLVED OXYGEN R3	3.4	4.3	5.6	5.8	4.1	3.6	3	2.4	1.8	1.3	0.9	0.4	1.1	1.8	2.6
ALKALINITY R3	900	921	930	912	903	870	863	851	841	836	800	763	748	796	767
MLSS R3	11704	11485	7520	11816	11500	12252	11956	11476	12048						
Effluent analysis															
COLOUR R3	0.57	0.59	0.53	0.6	0.6	0.58	0.55	0.53	0.49	0.39	0.32	0.3	0.29	0.39	0.37
AMMONIA R3	0.3	0.1	0.1	0.08	0.06	0.1	0.07	0.1	0.13	0.14	0.36	0.34	0.33	0.28	0.22
NITRATE R3	16.56	16.94	9.78	5	4.22	3.84	3.56	3.32	3.08	1.9	1.52	2.28	3.87	6.68	7.44
NITRITE R3	4.6	3.1	2.5	1.3	1.5	1.5	1.6	1.2	1.1	1.3	1.5	1.7	0.83	0.76	0.29
CHLORIDE R3	2798	2858	2796	2668	3085	2948	2685	2799	2816	3177	3244	3009	3303	3496	3422
SULPHATE R3	1856	1806	1688	1539	2089	1995	1794	2112	2122	1954	1739	1530	1585	1666	1723
TOC R3	82.4	86.4	93.4	90.3	88.4	82.7	72.6	73	78.7	81.2	122.7	132.4	139.8	104.8	96.8
ANILINE R3	0.37	0.23	0.17	0.2	0.17	0.13	0.47	0.33	0.45	1.11	1.98	1.58	0.94	0.66	0.34
BENZOTHIAZOLE R3	0.05	0.11	0.01	0.12	0.1	0.11	0.12	0.15	0.14	2.21	2.76	3.17	5.21	3.71	1.91
FLECTOL A R3	15.35	12.86	11.76	5.33	11.3	19.45	31.19	42.23	37.1	38.86	24.23	28.67	21.33	27.82	24.12
DMK R3	31.56	24.08	39.38	38.71	26.66	33.25	51.72	44.09	35.15	57.77	57.17	59.64	41.23	51.33	42.28

Time/days	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Biological reactor analysis															
pH REACTOR R3	7.93	7.93	7.9	7.83	7.79	7.76	7.92	8.11	8.09	8	8	7.93	7.95	7.9	7.93
Temp Deg C R3	27.2	26.6	25.6	22.3	24.2	23.4	23.3	23.3	23.3	25	25.7	25	23.5	23.7	23.1
DISSOLVED OXYGEN R3	3.3	2.5	2.6	2.6	2.6	2.4	2.6	2.4	2.7	2.3	1.9	2.1	2.7	2.4	2.5
ALKALINITY R3	786	801	811	829	835	851	880	896	915	662	675	718	738	715	736
MLSS R3															
Effluent analysis															
COLOUR R3	0.38	0.59	0.63	1	1	0.91	0.86	0.95	0.9	0.84	0.89	0.92	0.98	1	1
AMMONIA R3	0.18	0.13	0.19	0.13	0.18	0.34	0.27	0.2	0.16	0.11	0.03	0.04	0.07	0.1	0.16
NITRATE R3	7.5	9.47	9.94	10.84	9.27	10.32	9.14	8.25	6.68	3.93	3.64	2.82	3.1	2.96	2.84
NITRITE R3	0.001	4.11	4.03	3.51	3.06	2.13	1.87	1.13	0.96	0.69	0.51	0.46	0.21	0.19	0.13
CHLORIDE R3	3401	2691	2727	2895	2961	3096	3116	3175	3131	3101	3035	3090	3393	3160	3219
SULPHATE R3	1755	1911	1863	1805	1855	1911	1923	1821	1895	1793	1790	1816	1827	1830	1792
TOC R3	90.8	62	50.1	52.7	61.9	79.8	66.2	53.8	50	43.3	45.2	56.8	61.4	67.8	62.6
ANILINE R3	0.05	0.01	0.06	0.09	0.01	0.11	0.2	0.23	0.13	0.1	0.17	0.25	0.19	0.11	0.06
BENZOTHIAZOLE R3	1.22	0.19	0.26	0.18	0.23	0.56	0.48	0.27	0.13	0.1	1.53	1.11	1.06	0.23	0.11
FLECTOL A R3	15.65	5.81	5	4.17	6.96	14.96	23.67	19.21	19	18.76	21.6	24.88	29.47	22.27	18.23
DMK R3	35.67	18.26	23.61	41.02	49.89	54.27	41.77	36.36	32.06	24.19	26.16	30	42.73	41.07	36.33

Time/days	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Biological reactor analysis															
pH REACTOR R3	7.9	7.88	7.88	7.86	7.8	7.89	7.96	7.92	7.99	8.03	8.08	8.1	8.14	8.08	8
Temp Deg C R3	22.9	22.7	22.6	22	22.4	22.6	22.5	23.1	22.9	22.5	23	22.8	22.4	22	21.7
DISSOLVED OXYGEN R3	2.3	2.4	2.2	2.5	2.8	2.3	2.6	2.4	2.3	2.7	2.6	2.3	2.6	2.2	2.6
ALKALINITY R3	628	613	638	651	683	682	693	673	691	713	736	749	733	705	688
MLSS R3															
Effluent analysis															
COLOUR R3	1.1	0.9	0.85	0.8	0.86	0.91	0.94	0.9	0.86	0.88	0.75	0.82	0.83	0.76	0.79
AMMONIA R3	0.13	0.09	0.09	0.14	0.17	0.23	0.41	0.26	0.19	0.28	0.19	0.14	0.19	0.15	0.11
NITRATE R3	3.26	3.44	3.86	4.22	4.48	4.16	3.8	3.86	4.16	4.06	3.92	3.64	3.52	3.26	3.38
NITRITE R3	0.96	0.71	0.31	0.09	0.4	0.03	0.08	0.14	0.1	0.18	0.13	0.12	0.15	0.1	0.11
CHLORIDE R3	3293	3201	3133	3095	3164	3116	3106	3066	3005	2931	2890	2953	3086	3184	3208
SULPHATE R3	1736	1799	1827	1863	1886	1891	1886	1927	2001	2035	1986	2105	2234	2100	2033
TOC R3	53.3	49.8	46.1	30.8	39.2	46.9	41.5	52.4	63.6	60.1	57.5	55.4	53.8	51.5	44.8
ANILINE R3	0.01	0.01	0.07	0.09	0.13	0.14	0.21	0.14	0.16	0.13	0.19	0.21	0.17	0.12	0.09
BENZOTHIAZOLE R3	0.31	0.26	0.23	0.18	0.23	0.29	0.16	0.16	0.19	0.27	0.33	0.27	0.23	0.31	0.27
FLECTOL A R3	23.62	17	6.11	2.15	3.11	4.62	12.98	17.66	23.62	17.66	12.34	8.51	3.69	2.17	3.11
DMK R3	39.07	41	49.39	56.48	55.45	48.76	49.06	45.21	43.46	40.13	49.67	43.78	39.78	43.57	45.92

Time/days	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
Biological reactor analysis															
pH REACTOR R3	7.96	7.82	7.93	7.82	7.76	7.71	7.86	7.94	7.85	7.8	7.76	7.86	7.94	7.99	7.92
Temp Deg C R3	21.9	21.6	22	22.3	22.7	22.1	21.7	21.3	21.3	21.6	21.9	22.2	22.6	23.1	22.7
DISSOLVED OXYGEN R3	2.2	2.5	2.1	1.9	1.9	1.7	1.9	2.3	2.5	2.9	2.6	2.3	2.1	2.1	2.4
ALKALINITY R3	672	633	611	600	581	563	539	524	502	498	486	480	453	441	419
MLSS R3															
Effluent analysis															
COLOUR R3															
AMMONIA R3	0.24	0.2	0.19	0.25	0.27	0.23	0.2	0.22	0.28	0.24	0.26	0.27	0.25	0.23	0.2
NITRATE R3															
NITRITE R3															
CHLORIDE R3															
SULPHATE R3															
TOC R3	46.8	39.9	41.7	42.6	40.1	36.9	37.4	38.1	36.7	35.9	37.2	36.4	38.1	35.7	36.9
ANILINE R3															
BENZOTHIAZOLE R3															
FLECTOL A R3															
DMK R3															

Time/days	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
Biological reactor analysis															
pH REACTOR R3	8.04	8.08	8.11	8.06	8.14	8.21	8.13	8.05	7.99	7.92	7.84	7.88	7.81	7.74	7.71
Temp Deg C R3	22.4	22.1	22.1	21.7	21.6	22	21.8	21.5	21.1	21.6	21.4	21.9	22.5	22.7	22.5
DISSOLVED OXYGEN R3	2.6	2.3	2.1	1.9	1.9	1.7	1.6	2.1	2.3	2.5	2.2	2.5	2.8	2.5	2.5
ALKALINITY R3	400	415	456	472	495	481	460	434	429	444	459	436	448	466	471
MLSS R3															
Effluent analysis															
COLOUR R3															
AMMONIA R3	0.17	0.18	0.22	0.24	0.26	0.29	0.32	0.29	0.27	0.23	0.26	0.22	0.2	0.23	0.25
NITRATE R3															
NITRITE R3															
CHLORIDE R3															
SULPHATE R3															
TOC R3	37.2	40.2	38.9	37.3	36.4	35.8	36.9	37.5	40.1	38.1	37.1	35.8	37.5	38.4	37.6
ANILINE R3															
BENZOTHIAZOLE R3															
FLECTOL A R3															
DMK R3															

Time/days	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
Biological reactor analysis															
pH REACTOR R3	7.79	7.85	7.8	7.72	7.69	7.86	7.8	7.83	7.85	7.92	7.98	7.84	7.88	7.76	7.7
Temp Deg C R3	22.4	22.7	22.4	22.1	22.4	22.6	22.3	22.8	22.5	22.3	21.8	21.6	21.8	21.9	21.8
DISSOLVED OXYGEN R3	2.6	2.3	2.3	2	2.1	2.2	2.4	2.1	2	2.3	2.5	2.4	2.6	2.3	2.1
ALKALINITY R3	463	453	432	439	475	489	500	531	511	533	508	529	503	489	508
MLSS R3															
Effluent analysis															
COLOUR R3															
AMMONIA R3	0.25	0.3	0.34	0.37	0.42	0.44	0.35	0.3	0.27	0.26	0.24	0.28	0.35	0.33	0.34
NITRATE R3															
NITRITE R3															
CHLORIDE R3															
SULPHATE R3															
TOC R3	37.6	38.2	37.2	36.4	37.1	38.3	37	37.5	36.9	36.1	35.6	35.4	37.9	38.6	37.1
ANILINE R3															
BENZOTHIAZOLE R3															
FLECTOL A R3															
DMK R3															

Time/days	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Biological reactor analysis															
pH REACTOR R3	7.81	7.87	7.9	7.92	7.96	7.9	7.94	8.01	7.94	7.98	7.86	7.79	7.85	7.81	7.88
Temp Deg C R3	22.1	22.5	22.6	22.3	22	22.3	22.3	22	21.9	21.7	22.1	22.3	22.4	22.5	22.5
DISSOLVED OXYGEN R3	2.5	2.3	2.3	2.2	2.3	2.1	2	2.3	2.3	2.1	2.3	2.3	2	2.1	2.3
ALKALINITY R3	519	503	526	513	500	515	528	509	498	513	532	515	535	511	532
MLSS R3															
Effluent analysis															
COLOUR R3															
AMMONIA R3	0.36	0.42	0.45	0.41	0.39	0.36	0.4	0.44	0.42	0.45	0.41	0.37	0.34	0.38	0.33
NITRATE R3															
NITRITE R3															
CHLORIDE R3															
SULPHATE R3															
TOC R3	36.4	35.8	35.1	36.4	37.6	37.8	36.9	36	35.9	36.5	37.3	38.1	38.9	37.2	36.8
ANILINE R3															
BENZOTHIAZOLE R3															
FLECTOL A R3															
DMK R3															

Appendix 2D Average data for all three bioreactors including min and max values

			Biological Reactor Average, Min and Max values						
	Biological Reactor R1			Biological Reactor R2			Biological Reactor R3		
	Average	Min	Max	Average	Min	Max	Average	Min	Max
Biological reactor analysis									
pH REACTOR	7.88	7.2	8.24	7.89	7.21	8.23	7.91	7.31	8.23
Temp Deg C	23.0	21	26.7	22.9	21	26.8	23.1	21.1	27.2
DISSOLVED OXYGEN	2.4	0.3	7.6	2.4	0	7.1	2.5	0.4	6.8
ALKALINITY	631	403	960	635	411	948	638	400	936
MLSS	11524	6284	13048	11529	8140	14544	11069	6496	13272
Effluent analysis									
COLOUR	0.82	0.23	1.24	0.77	0.29	1.28	0.77	0.29	1.1
AMMONIA	0.23	0.06	0.56	0.23	0.04	0.56	0.23	0.03	0.45
NITRATE	6.96	1.5	16.46	6.59	1.66	16.21	6.66	1.52	16.94
NITRITE	1.17	0.01	7.4	1.21	0.01	6.2	1.36	0.001	7.3
CHLORIDE	3031	2361	3876	3080	2367	3873	3070	2332	3955
SULPHATE	1865	1371	2239	1900	1586	2196	1879	1530	2234
TOC	51.75	34.6	147.4	51.7	33.6	137.2	51.7	30.8	139.8
ANILINE	0.40	0.02	2.79	0.44	0.03	2.54	0.40	0.01	1.98
BENZOTHIAZOLE	1.01	0.08	9.76	0.83	0.05	5.48	0.69	0.01	5.21
FLECTOL A	15.40	0.51	39.24	16.76	2.15	42.23	16.76	2.15	42.23
DMK	44.73	17.11	73.93	46.26	16.11	73.21	41.96	18.26	76.74

Appendix 2E Full analytical data for bio reactor R3 during synthetic feed analysis

Time/days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Biological reactor analysis															
pH REACTOR R3	7.35	7.38	7.34	7.39	7.42	7.42	7.37	7.34	7.46	7.51	7.5	7.61	7.54	7.5	7.51
Temp Deg C R3	24.3	24.9	25.3	25.4	25.6	24.8	24.4	24	23.8	23.5	23.8	24.2	24.7	24.9	25.3
DISSOLVED OXYGEN R3	1.9	2.1	2.1	1.9	1.9	2.1	2.3	2.1	2.1	1.9	1.7	1.7	1.6	1.8	1.9
ALKALINITY R3	425	475	473	465	453	442	453	461	468	455	449	440	422	437	444
MLSS R3	18353	18744	18882	19045	18756	18623	18532	18615	18715	18522	18618	18756			
Effluent analysis															
COLOUR R3	0.05	0.08	0.04	0.02	0.05	0.06	0.04	0.03	0.05	0.03	0.08	0.04	0.05	0.08	0.11
AMMONIA R3	0.11	0.21	0.25	0.18	0.22	0.26	0.31	0.24	0.18	0.11	0.14	0.18	0.2	0.16	0.24
NITRATE R3	0.02	0.03	0.01	0	0	0.01	0.03	0.01	0.03	0.02	0.05	0.04	0.02	0	0
NITRITE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
CHLORIDE R3	2355	2373	2391	2356	2321	2348	2400	2456	2415	2378	2365	2371	2391	2380	2365
SULPHATE R3	1455	1460	1472	1463	1432	1475	1435	1463	1456	1445	1463	1456	1448	1450	1458
TOC R3	16.66	16.88	17.21	17.36	17.85	17.05	17.62	17.01	16.55	16.45	16.02	16.34	16.66	16.46	16.88
ANILINE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
BENZOTHIAZOLE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
FLECTOL A R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
DMK R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Time/days	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Biological reactor analysis															
Time/days															
pH REACTOR R3	7.53	7.49	7.47	7.45	7.51	7.48	7.46	7.44	7.43	7.43	7.51	7.58	7.52	7.52	7.55
Temp Deg C R3	25.4	25.7	25.2	25	24.8	24.6	24.7	24.9	24.6	24.3	24	23.7	23.4	23.9	24.3
DISSOLVED OXYGEN R3	2.1	2.4	2.3	2.4	2.1	2.2	2	1.9	1.7	1.6	1.5	1.7	1.9	2.1	2
ALKALINITY R3	452	455	461	468	458	455	447	449	455	465	466	461	463	460	461
MLSS R3															
Effluent analysis															
COLOUR R3	0.12	0.11	0.09	0.06	0.07	0.05	0.05	0.08	0.05	0.07	0.03	0.04	0.05	0.08	0.06
AMMONIA R3	0.26	0.31	0.24	0.26	0.21	0.22	0.17	0.23	0.24	0.26	0.23	0.22	0.23	0.24	0.28
NITRATE R3	0.01	0.02	0.04	0.06	0.04	0.04	0.07	0.05	0.04	0.06	0.03	0.02	0.05	0.04	0.02
NITRITE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
CHLORIDE R3	2341	2298	2271	2246	2271	2250	2236	2221	2248	2267	2260	2249	2269	2278	2290
SULPHATE R3	1463	1467	1473	1481	1476	1464	1470	1465	1469	1473	1466	1469	1463	1460	1471
TOC R3	16.42	17	17.31	17.2	16.88	16.45	16.05	15.89	15.75	16.21	16.22	16.37	16.2	16.41	16.8
ANILINE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
BENZOTHIAZOLE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
FLECTOL A R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
DMK R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Time/days	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Biological reactor analysis															
pH REACTOR R3	7.45	7.4	7.38	7.36	7.39	7.41	7.45	7.47	7.43	7.47	7.45	7.41	7.44	7.42	7.4
Temp Deg C R3	23.9	24.2	24.6	24.6	24.9	24.5	24.2	23.7	23.6	23.2	23.5	23.8	24.1	24.4	24.1
DISSOLVED OXYGEN R3	1.8	2	2.2	2.4	2.1	2.2	2.4	2.5	2.3	2.4	2.6	2.4	2.4	2.1	2.3
ALKALINITY R3	459	453	457	455	462	466	473	470	471	476	468	460	466	456	458
MLSS R3															
Effluent analysis															
COLOUR R3	0.09	0.08	0.06	0.04	0.06	0.05	0.08	0.06	0.08	0.1	0.11	0.1	0.1	0.11	0.13
AMMONIA R3	0.25	0.28	0.3	0.33	0.34	0.33	0.29	0.24	0.26	0.25	0.22	0.24	0.25	0.23	0.24
NITRATE R3	0.05	0.06	0.04	0.04	0.02	0.03	0.05	0.04	0.04	0.07	0.04	0.05	0.03	0.05	0.04
NITRITE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
CHLORIDE R3	2275	2260	2249	2258	2281	2290	2295	2310	2321	2230	2283	2275	2291	2276	2265
SULPHATE R3	1473	1477	1472	1468	1466	1470	1472	1469	1466	1461	1460	1463	1466	1460	1461
TOC R3	16.18	16.23	16.13	16.2	16.28	16.8	16.21	16.16	16.23	16.09	16.04	15.88	15.76	15.58	15.93
ANILINE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
BENZOTHIAZOLE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
FLECTOL A R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
DMK R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Time/days	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
Biological reactor analysis															
pH REACTOR R3	7.52	7.49	7.47	7.43	7.46	7.5	7.51	7.49	7.46	7.43	7.4	7.38	7.34	7.36	7.41
Temp Deg C R3	24.6	24.8	24.5	24.3	24.7	25.2	25.4	25	24.7	24.5	24.6	24.3	23.9	23.7	23.4
DISSOLVED OXYGEN R3	2.2	2.2	2.1	2.3	2.1	2.2	2.1	2.3	2.4	2.6	2.3	1.9	1.7	1.9	1.7
ALKALINITY R3	456	450	448	453	455	459	451	458	463	463	475	472	468	466	456
MLSS R3															
Effluent analysis															
COLOUR R3	0.08	0.09	0.11	0.11	0.09	0.07	0.1	0.13	0.12	0.09	0.08	0.11	0.1	0.08	0.06
AMMONIA R3	0.25	0.22	0.19	0.18	0.21	0.17	0.22	0.19	0.23	0.21	0.22	0.24	0.26	0.25	0.27
NITRATE R3	0	0	0	0.02	0.01	0.03	0.02	0.04	0.03	0.01	0.03	0.05	0.04	0.06	0.07
NITRITE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
CHLORIDE R3	2312	2322	2336	2318	2341	2333	2311	2329	2300	2313	2323	2346	2305	2286	2264
SULPHATE R3	1466	1461	1460	1459	1455	1457	1450	1455	1460	1463	1459	1464	1468	1472	1475
TOC R3	16.77	16.81	16.99	16.84	16.58	16.71	16.89	16.64	16.23	16.17	16.08	16.09	16.15	16.06	16
ANILINE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
BENZOTHIAZOLE R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
FLECTOL A R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
DMK R3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Appendix 2F Average, min and max for bioreactor R3 during synthetic feed analysis

Average,Min & Max for Bioogical reactor R3			
	Average	Min	Max
Biological reactor analysis			
pH REACTOR R3	7.45	7.34	7.61
Temp Deg C R3	24.4	23.2	25.7
DISSOLVED OXYGEN R3	2.1	1.5	2.6
ALKALINITY R3	458	422	476
MLSS R3	18680	18353	20274
Effluent analysis			
COLOUR R3	0.07	0.02	0.13
AMMONIA R3	0.23	0.11	0.34
NITRATE R3	0.03	0	0.07
NITRITE R3			
CHLORIDE R3	2309	2221	2456
SULPHATE R3	1463	1432	1481
TOC R3	16.48	15.58	17.85
ANILINE R3	N.D	N.D	N.D
BENZOTHIAZOLE R3	N.D	N.D	N.D
FLECTOL A R3	N.D	N.D	N.D
DMK R3	N.D	N.D	N.D

Appendix 2G Chronological record for bioreactor R1, year 1 and subsequent years

Activity	Set up test reactors	Leak test reactors	Prime with biomass	Acquire baseline data	Determine Vitox feed composition	Determine base matrix composition for use in R3	Evaluation of memory effect	Continued use as control reactor for all future experimental work
Jan Wk1								
Wk2								
Wk3								
Wk4								
Feb Wk1								
Wk2								
Wk3								
Wk4								
Mar Wk1								
Wk2								
Wk3								
Wk4								
Apr Wk1								
Wk2								
Wk3								
Wk4								
May Wk1								
Wk2								
Wk3								
Wk4								
June Wk1								
Wk2								
Wk3								
Wk4								
July Wk1								
Wk2								
Wk3								
Wk4								
Aug Wk1								
Wk2								
Wk3								
Wk4								
Sept Wk1								
Wk2								
Wk3								
Wk4								
Oct Wk1								
Wk2								
Wk3								
Wk4								
Nov Wk1								
Wk2								
Wk3								
Wk4								
Dec Wk1								
Wk2								
Wk3								
Wk4								

Reactor continued to be used as a control unit for the remainder of the research project.

Appendix 2H Chronological record for bioreactor R2, year 1

	Activity	Set up test reactors	Leak test reactors	Prime with biomass	Aquire baseline data	Determine Vitox feed composition	Determine base matrix composition, for use in R3	Intermitant use as a control reactor Vs R1 and R3	Nitrification / Denitrification study
Jan Wk1									
Wk2									
Wk3									
Wk4									
Feb Wk1									
Wk2									
Wk3									
Wk4									
Mar Wk1									
Wk2									
Wk3									
Wk4									
Apr Wk1									
Wk2									
Wk3									
Wk4									
May Wk1									
Wk2									
Wk3									
Wk4									
June Wk1									
Wk2									
Wk3									
Wk4									
July Wk1									
Wk2									
Wk3									
Wk4									
Aug Wk1									
Wk2									
Wk3									
Wk4									
Sept Wk1									
Wk2									
Wk3									
Wk4									
Oct Wk1									
Wk2									
Wk3									
Wk4									
Nov Wk1									
Wk2									
Wk3									
Wk4									
Dec Wk1									
Wk2									
Wk3									
Wk4									

Appendix 2I Chronological record for bioreactor R2, years 2–4

	Activity	Intermittent use as a control reactor for R1 and R3	Experiment to determine the memory effects of the biomass	Investigation into colour formation	Intermittent use as a control reactor for R1 and R3	Intermittent use as a control reactor for R1 and R3
Jan Wk1						
Wk2						
Wk3						
Wk4						
Feb Wk1						
Wk2						
Wk3						
Wk4						
Mar Wk1						
Wk2						
Wk3						
Wk4						
Apr Wk1						
Wk2						
Wk3						
Wk4						
May Wk1						
Wk2						
Wk3			Reacclimation to Vitox feed			
Wk4						
June Wk1						
Wk2						
Wk3						
Wk4						
July Wk1						
Wk2						
Wk3						
Wk4						
Aug Wk1			Reacclimation to Vitox feed			
Wk2						
Wk3						
Wk4						
Sept Wk1						
Wk2						
Wk3						
Wk4						
Oct Wk1						
Wk2						
Wk3						
Wk4				Reacclimation to Vitox feed		
Nov Wk1						
Wk2						
Wk3						
Wk4						
Dec Wk1						
Wk2						
Wk3						
Wk4						

KEY: 2nd year | 3rd year | 4th year

Appendix 2J Chronological record for bioreactor R3, year 1

	Activity	Set up test reactors	Leak test reactors	Prime with biomass	Acquire baseline data	Determine Vitox feed composition	Determine base matrix composition	Washout preparation of sludge for future experimental work	Thiazolic compounds metabolisation studies	Analytical method development
Jan Wk1										
Wk2										
Wk3										
Wk4										
Feb Wk1										
Wk2										
Wk3										
Wk4										
Mar Wk1										
Wk2										
Wk3										
Wk4										
Apr Wk1										
Wk2										
Wk3										
Wk4										
May Wk1										
Wk2										
Wk3										
Wk4										
June Wk1										
Wk2										
Wk3										
Wk4										
July Wk1										
Wk2										
Wk3										
Wk4										
Aug Wk1										
Wk2									BTH	
Wk3										
Wk4								Washout		
Sept Wk1										
Wk2										
Wk3										
Wk4										
Oct Wk1									BTOH	
Wk2										
Wk3								Washout		
Wk4										
Nov Wk1										
Wk2									AB	
Wk3										
Wk4								Washout		
Dec Wk1										
Wk2										
Wk3								Holiday		
Wk4										

Appendix 2K Chronological record for bioreactor R3, years 2–4

	Activity	Thiazolic compounds metabolisation studies	Analytical method development	Thiazolic compounds metabolisation studies	Thiazolic compounds metabolisation studies	Analytical method development	Non-thiazolic substrate metabolisation studies	Analytical method development	Non-thiazolic substrate metabolisation studies	Analytical method development
Jan Wk1		MBT			MBT/ BTOH/ BTSA					
Wk2									PHBA	
Wk3		Washout			Washout					
Wk4										
Feb Wk1									Washout	
Wk2										
Wk3		MBS			BTH/ BTOH/ MBT					
Wk4									Methyl parabens	
Mar Wk1					Washout					
Wk2										
Wk3		Washout							Washout	
Wk4										
Apr Wk1					BTH/ BTOH/ BTSA/ MBT					
Wk2										
Wk3					Washout					
Wk4				MBT/ BTSA						
May Wk1										
Wk2				Washout						
Wk3							TMQ			
Wk4										
June Wk1				BTOH/ BTSA			Washout			
Wk2										
Wk3				Washout						
Wk4										
July Wk1										
Wk2				BTH/ BTOH			DPG			
Wk3										
Wk4				Washout			Washout			
Aug Wk1										
Wk2										
Wk3				MBT/ BTOH			PVI literature search - no experimental work			
Wk4										
Sept Wk1				Washout						
Wk2										
Wk3										
Wk4										
Oct Wk1				No Experiment - Holidays*						
Wk2							No Experiment - Holidays*			
Wk3										
Wk4				MBT/ BTH						
Nov Wk1							PVI			
Wk2				Wash-out						
Wk3										
Wk4				Extended washout prior to ternary substrate studies and Holidays			Extended washout due to Holiday break			
Dec Wk1										
Wk2										
Wk3										
Wk4										

KEY: 2nd year | 3rd year | 4th year

CHAPTER 3
EFFECT OF THIAZOLES ON NITRIFYING BACTERIA

3.1 Nitrification

As outlined in Chapter 1, nitrification is traditionally defined as the oxidation of NH_4^+ via NO_2^- to NO_3^- with O_2 as the terminal electron acceptor. The oxidation of NH_3 to NO_2^- is due mainly to the action of two groups of autotrophic organisms typified by the genera *Nitrosomonas* and *Nitrobacter* (Koops *et al.*, 1991). The ammonia oxidisers, such as *Nitrosomonas*, oxidise NH_3 to NO_2^- in two steps: the first is oxidation of NH_3 to NH_2OH (hydroxylamine) with a second step involving oxidation of NH_2OH to NO_2^-. The nitrite oxidisers, such as *Nitrobacter*, are responsible for oxidation of NO_2^- to NO_3^- (Figure 3.1).

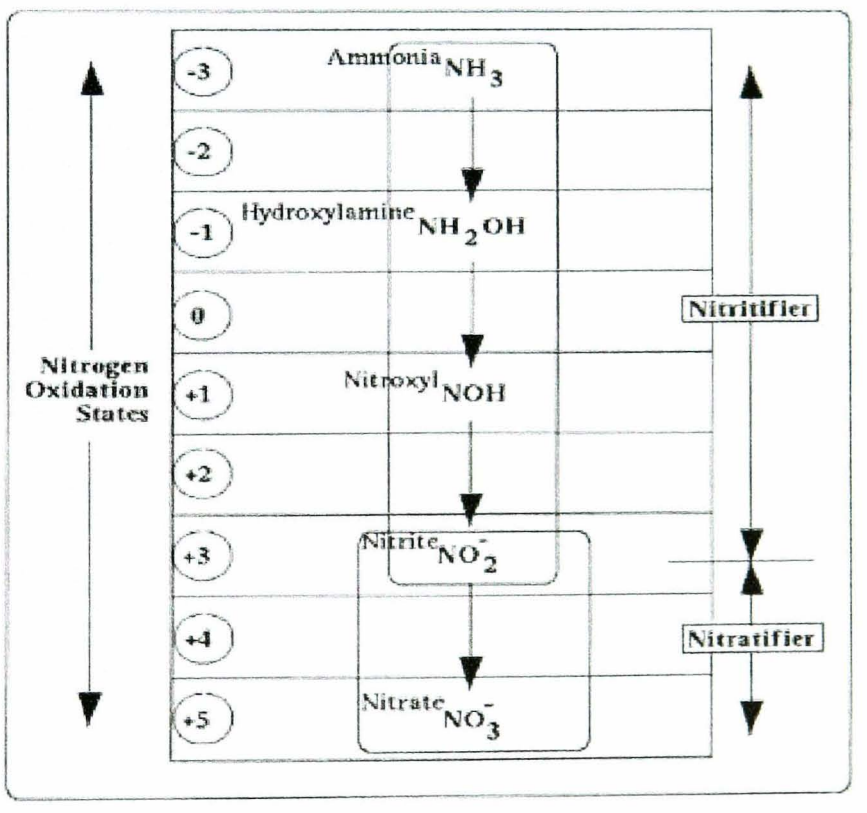


Figure 3.1 Currently accepted pathway for nitrification (Suzuki *et al.*, 1974).

3.2 Denitrification

Ammonia oxidisers are capable of reversing what is considered to be their natural reaction process by reducing NO_2^- even in the presence of oxygen (Hooper, 1989; van de Graaf *et al.*, 1990). A number of studies have reported that autotrophic nitrifiers are able to reduce

NO_2^- to NO, N_2O or N_2 under oxygen limitation (Poth and Focht, 1985; Poth, 1986; Remde and Conrad, 1990; Bock *et al.*, 1995). This process is known as aerobic denitrification with the end product of the reaction being dependent on the organisms and the presence of a suitable electron acceptor, bacterial grouping and genetics. However, denitrification on the plant scale usually relies on the anoxic process.

3.3 Bacterial grouping and genetics

The group of nitrifying genera consists of two distinctly different subsets, based on whether substrate consumption is either ammonium or nitrite ion. Table 3.1 depicts the breakdown of the associated bacterial genera and species, covering a total of eight separate nitrifying bacteria which include five species of ammonia oxidisers (often called 'nitritifiers'), and three nitrite-oxidisers ('nitratifiers').

Table 3.1 Bacterial genera of nitrifiers.

Nitritifiers or ammonia-oxidisers
Nitrosomonas europaea
Nitrospira brientsis
Nitrosococcus nitrosus
Nitrosococcus oceanus
Nitrosolobus multiformis
Nitratifiers or nitrite-oxidisers
Nitrobacter winogradsky
Nitrospina gracilis
Nitrococcus mobilis

3.4 Nitrification/denitrification

Even after a full century of intensive study, microbiologists still do not fully understand just how the nitrification sequence begins. There is some confusion over the 'nitrifiers' initial substrate form. Many modern textbooks infer that nitrification starts from ionised ammonium-nitrogen. The work of Suzuki *et al.* (1974), however, strongly suggested that the lead 'nitrifying' organism used un-ionised ammonia NH_3, in a gaseous state, based on an analysis of the organism's response to elevated substrate levels.

Assuming that ammonia is, in fact, the starting point, Figure 3.1 shows a complete overview of the currently accepted pathway for the various nitrogen transformations along the proposed pathway of nitrification. The various intermediates involved in this sequence are very rarely present at measurable levels. However, under certain conditions, it is conceivable that the process could become disrupted, resulting in elevated levels developing for one or more of the intermediates. A variety of stress-inducing conditions might promote such a disruption, such as oxygen deprivation or the presence of toxic (inhibitory) substances or significant changes in pH or salinity.

It is also possible to disrupt the nitrification process through excess sludge wastage: in this case the cause of disruption is due to 'washout' of the nitrifier population. It is known that nitrifier organisms are slow growing compared to other bacterial strains. As a consequence excessive sludge wastage will wash out the nitrifiers, thus reducing the total nitrifier population. The sludge biomass is a homogeneous system and if the proportion of sludge wasted is greater than the proportional increase in nitrifiers then it becomes difficult or impossible to establish a stable nitrifier population.

3.5 Nitrification inhibition

The phenomenon of nitrification inhibition has been known for a long time. Intensive research was carried out at the beginning of the 1960s in the field of nitrification and the inhibition of the nitrification process (Downing *et al.*, 1964; Knowles *et al.*, 1965; Tomlinson *et al.*, 1966). It became apparent during this research that water treatment plants treating industrial wastewater were affected by nitrification inhibition. A wealth of research has been carried out during the years since Downing began his work back in 1964. A lot of this work has concentrated on the specific mechanisms and rates of both nitrification and denitrification processes, and in particular how these processes are inhibited by the presence of various substrates.

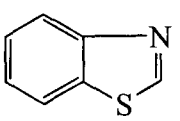
It was therefore not the aim of this research to repeat this type of investigation into the nitrification and denitrification processes, but rather to investigate the effects of pH on the Ruabon biomass with respect to nitrification and how this biomass reacted to the presence

of elevated levels of benzothiazole and benzothiazole derivatives in the feed to the biological reactor. These compounds were selected because of their importance to the Flexsys operation at the Ruabon site, where a range of benzothiazole derivatives are manufactured for use in the rubber processing industry.

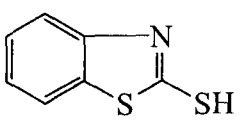
Various workers in the field have shown that certain chemicals exert inhibitory effects on activated sludge systems. It is well known for instance that 2-mercaptobenzothiazole is generally toxic to bacteria and interferes with the nitrification process (Tomlinson *et al.*, 1966; Czechowski and Rossmore, 1981; Knapp *et al.*, 1982; De Vos *et al.*, 1993).

3.6 Rubber chemicals (benzothiazole and derivatives)

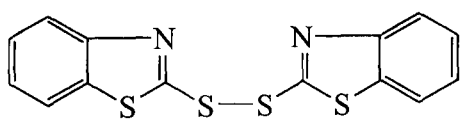
The Flexsys Rubber Chemicals site at Ruabon, North Wales, UK, manufactures a wide range of rubber chemicals for the tyre and non-tyre rubber processing industries. Many of these are based on the benzothiazole structure (Figure 3.2).



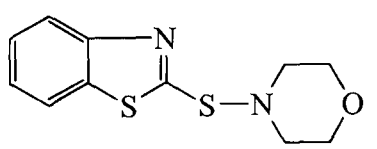
benzothiazole



2-mercaptobenzothiazole



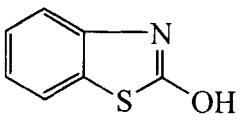
2,2'-dithiobis(benzothiazole)



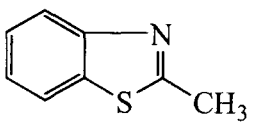
2-morpholinothiobenzothiazole

Figure 3.2 Benzothiazole derivatives manufactured by Flexsys at Ruabon.

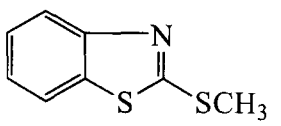
There are also a number of associated compounds of the same family which are not manufactured intentionally but are known to be generated within the various processes operated on the site (Figure 3.3).



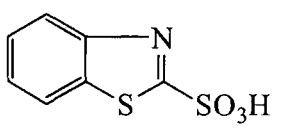
2-hydroxybenzothiazole (BTOH)



2-methylbenzothiazole (MeBTH)



2-methylmercaptobenzothiazole (MeMBT)



benzothiazole-2-sulphonic acid (BTSA)

Figure 3.3 Other benzothiazole derivatives produced at the Flexsys site.

The site also produces other compounds that are utilised in the rubber processing industry. These are known not to cause problems with the wastewater treatment plant at the Ruabon site. Accordingly these compounds are designated 'soft compounds' as they are nearly all fully degraded within the biological process. Flectol 'A' monomer is an exception, being difficult to degrade, but is not considered to be inhibitory towards the biomass.

The following compounds were used as sole substrates and in various combinations with each other to observe any adverse effects at various concentrations: BTH, MBT, BTOH and BTSA. One other parameter was investigated for any adverse effects on the nitrification process and that was pH, to show that the current biomass was behaving according to literature claims.

3.7 Methodology

In order to assess how pH affects the biomass and its ability to nitrify, a pH adjusted feed was prepared using normal feed obtained from the control room of the main wastewater treatment plant. The normal pH range for 'Vitox' feed is between 10 and 11 pH, but for this experiment the pH was adjusted using strong hydrochloric acid to lower the pH to 6.0. A similar batch of feed was obtained for the control reactor for comparison during the experiment and to ensure no other unknown effects were causing any observed loss of nitrification.

The indicator parameter to show any loss of nitrification is ammonia concentration in the effluent from the biological reactor and this was determined on a daily basis using an Orion ion meter with a gas sensing ammonia probe. The pH of the biological reactor was recorded at the same time the sample was taken for ammonia determination, pH being measured using a hand held HANNA Aqua check portable pH probe.

The normal operating pH of the reactor varied broadly between pH 7 and 8, with a mean of 7.8. This also happens to be within the cited optimum for nitrification to proceed (Bock *et al.*, 1986). The pH is regulated by the natural buffering capacity of the system; this is commonly known as the alkalinity of the biological reactor and is controlled by the bicarbonate-carbonate equilibrium.

The control was kept near constant in terms of pH allowing for natural variation, while the reactor being fed the low pH feed was adjusted to an acid pH. The pH and ammonia levels were monitored throughout the experiment while the pH was lowered and then brought back to normal levels *ca.* 7.8. The reactor was allowed to stabilise for a few days before the procedure was repeated.

3.8 Results and discussion

The results are presented in both tabular form (Tables 3.2 and 3.3) and as a chart (Figure 3.4) for ease of interpretation. The two tables contain the raw data for the experimental runs: Table 3.2 gives the data for the first run of the experiment while Table 3.3 gives the data for the repeat experiment. The chart shows very clearly how the lowering of pH had a significant effect on the biomass and caused the system to stop nitrifying. However, on restoring the pH the biomass soon recovered and nitrification re-established itself.

The results and graph clearly show how pH has a significant impact on the nitrifying ability of the organisms at extremes of pH. It is interesting to note that the biomass appears to develop an ability to continue with the nitrification process at lowered pH following a period of low pH. This may be due to acclimation or simply the effect of other organisms carrying out the process of nitrification.

The system did not recover any more quickly the second time after being run at an acid pH. This would have been expected if it were a case of acclimation. Accordingly it would be a fair assumption to attribute the continuing nitrification to other organisms present that are capable of nitrifying at the lower pH. A second possibility also exists, one in which the low pH prevents the degradation of nitrogenous material to produce ammonia within the biological reactor.

Table 3.2

Time/days	mg/l Ammonia +/– 0.02 mg/l	pH
1	0.09	7.64
2	0.11	7.47
3	0.12	7.50
4	0.08	7.52
5	0.14	7.51
6	0.12	7.53
7	0.15	7.58
8	0.04	7.54
9	0.09	7.49
10	0.16	7.52
11	0.14	7.33
12	0.16	6.92
13	11.57	4.84
14	28.40	3.76
15	35.40	3.82
16	33.80	4.78
17	21.60	6.37
18	11.35	7.11
19	3.48	7.28
20	1.17	7.48
21	0.07	7.62
22	0.04	7.59
23	0.07	7.57

Table 3.3

Time/days	mg/l Ammonia +/– 0.02 mg/l	pH
24	0.04	7.61
25	0.04	7.40
26	0.06	7.42
27	0.07	7.32
28	0.06	7.32
29	0.07	7.36
30	1.47	6.92
31	4.76	5.82
32	12.56	4.16
33	18.67	3.90
34	21.6	4.61
35	9.36	5.91
36	4.13	6.80
37	0.84	7.20
38	0.16	7.31
39	0.07	7.48
40	0.08	7.57
41	0.06	7.65
42	0.08	7.61
43	0.04	7.53
44	0.05	7.59
45	0.05	7.68
46	0.06	7.60
47	0.03	7.62
48	0.04	7.58
49	0.08	7.55
50	0.11	7.59
51	0.12	7.63

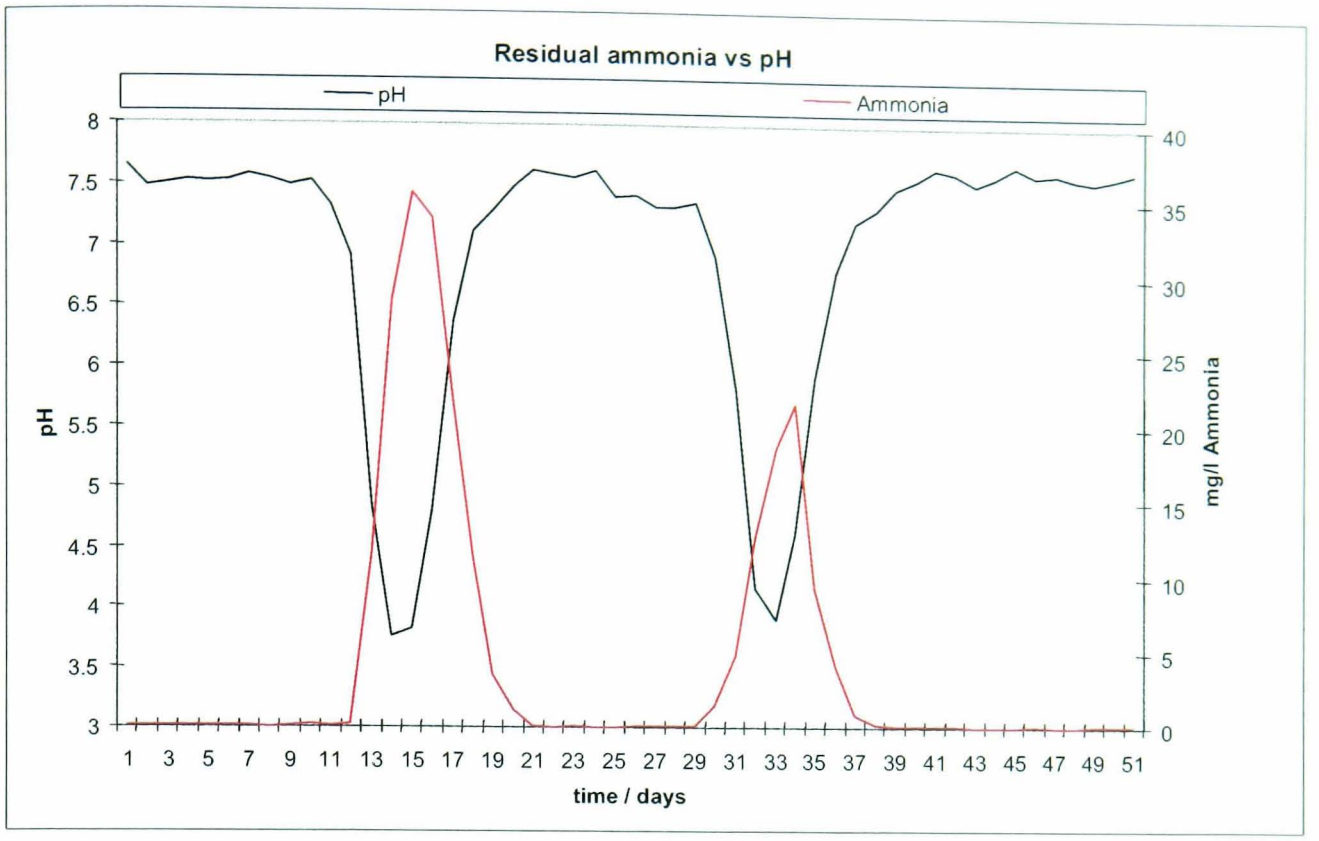


Figure 3.4 Residual ammonia vs pH.

There are two other factors to consider here apart from the pH effect on the bacteria themselves although these two parameters are linked to pH.

Consider the bicarbonate-carbonate equilibrium and how this is related to the cell biology of the various nitrifying populations and other organisms that utilise carbon from bicarbonate for new cell growth. At extremes of pH the carbonate in the form of HCO_3^{2-} is not available to the organisms. The chemistry of the bicarbonate-carbonate equilibrium is as follows:

$$CO_2 + H_2O \underset{\text{rate limiting step}}{\rightleftharpoons} H_2CO_3 \quad \text{<less than 1\% carbonic acid}$$

$$H_2CO_3 \underset{\text{fast}}{\rightleftharpoons} H^+ + HCO_3^- \quad \text{bicarbonate ion}$$

$$HCO_3^- \underset{\text{fast}}{\rightleftharpoons} H^+ + CO_3^{2-} \quad \text{carbonate ion}$$

$$\longleftarrow \text{acidic} \qquad \text{basic} \longrightarrow$$

The direction of the equilibrium is shifted by pH, acidic to the left, basic to the right.

Figure 3.5 is a graphical representation of this equilibrium.

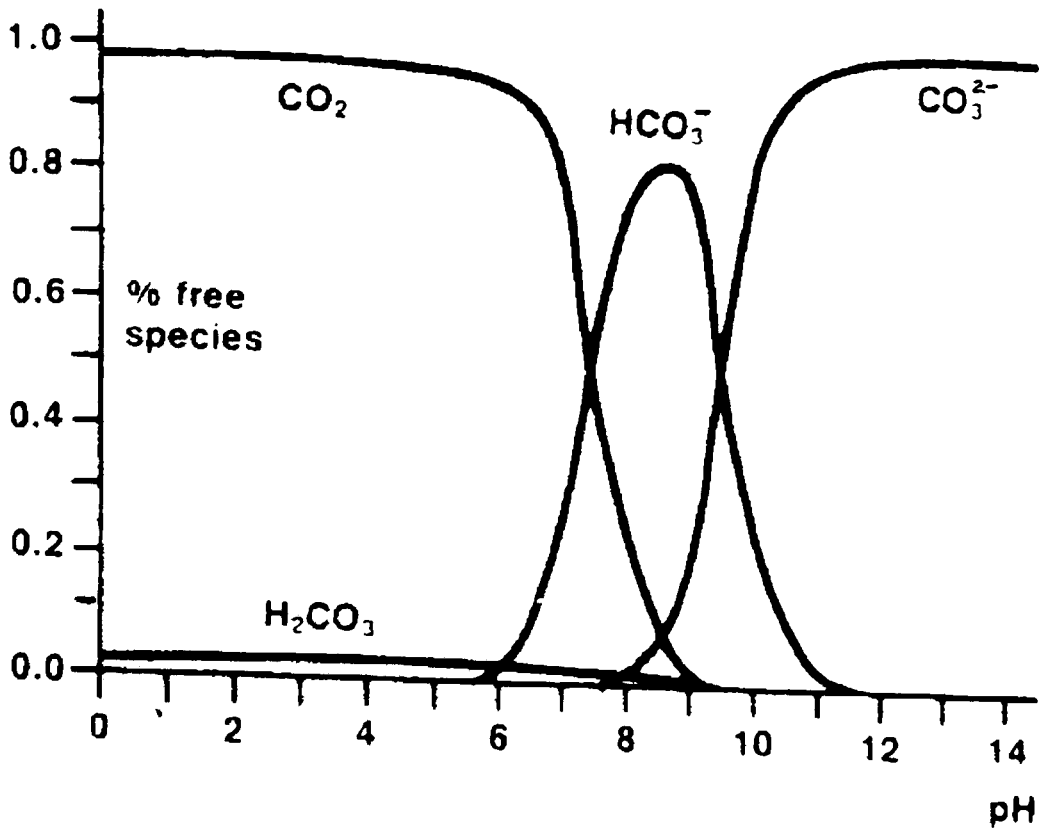


Figure 3.5 Fraction of carbonate ions, bicarbonate ions and carbon dioxide as a function of pH.

The graph shows how important pH is in maintaining this equilibrium since the pH determines the concentration of HCO_3^- present:

$$\mathbf{CO_2 + 2H_2O} \longleftarrow HCO_3^- + OH^- \longrightarrow H_2O + CO_3^{2-}$$

At low pH (extra H^+ present) the reaction is driven to the left and free CO_2 predominates:

$$H_2O + CO_2 \longleftarrow \mathbf{H^+ + HCO_3^-}$$

At high pH (extra OH^- present) the reaction is driven to the right and CO_3^{2-} predominates:

$$\mathbf{OH^- + HCO_3^-} \longrightarrow H_2O + CO_3^{2-}$$

Consider again the original point that a lowering of pH interferes with the nitrification process, as indicated by the rise in residual levels of ammonia. We can see from the above chemistry that this lowering of pH will affect the bicarbonate-carbonate equilibrium and more specifically the effect on the bicarbonate availability for (a) biological utilisation for new cell growth and (b) as a buffering component of the system.

It is the second point here that comes into play when nitrification proceeds, in that the conversion of ammonia to nitrite via nitrification generates H^+. This acidity would

normally be neutralised by the natural buffering capacity of the system, according to the equations above, and so maintain the equilibrium in keeping with Le Chatelier's principle.

It is obvious that in the case of pH it is not a simple situation that inhibits nitrification, *i.e.* interference of biological activity through change in environment pH, which obviously affects the fundamental performance of all bacteria at the molecular level. It is also the added effects of the shift in the bicarbonate-carbonate equilibrium and how this loss of bicarbonate availability has an additive effect on the inhibition of nitrification. Firstly, there is a reduced availability of bicarbonate carbon for new cell growth and secondly, this loss of the buffering capacity of the system allows the build up of acidity which further exacerbates the problem. The ammonia in the system can be ruled out as a contributory substance in the inhibition process, since the Ruabon biomass can treat levels in excess of 50 mg/l ammonia, which is in contradiction to some of the claims by other workers such as Anthonisen *et al.* (1976) and Abeling and Seyfried (1992).

Control of biological reactor pH (hydroxide alkalinity) and alkalinity (carbonate–bicarbonate) are clearly two of the more fundamental parameters if successful nitrification is to be maintained. Hydroxide alkalinity inhibits how the biomass performs while the carbonate-bicarbonate alkalinity can affect bacterial growth. This is not new, it is well researched and reported.

3.9 Chemical effects of thiazoles on nitrification

The studies of how chemical effects on nitrification occur were conducted using a specially prepared biomass. As described in Chapter 2, the biomass of reactor R3 was treated to remove all traces of rubber chemicals and associated metabolites. It was during this preparation step that an interesting and quite contradictory fact came to light.

According to the research of Anthonisen *et al.* (1976), the non-ionised forms of ammonia (NH_3) and nitrous acid (HNO_2) have an inhibitory effect on both the *Nitrosomonas* and *Nitrobacter* bacteria. Abeling and Seyfried (1992) reported that concentrations of 1–5 mg/l NH_3 inhibited nitratation but not the nitritation.

It was observed that the biomass of reactor R3 was fully nitrifying incoming feed at levels of 75 mg/l NH_3 with no apparent indication of inhibition. This is based on the residual ammonia analysis of effluent from the biological reactors. Furthermore there was sufficient alkalinity present that the system pH was maintained at all times in the region of 7.5–8.0. In fact the system was achieving an ammonia removal of over 99%, which is totally contradictory to the findings of Anthonisen *et al.* (1976) and Abeling and Seyfried (1992). Even in studies by Wong-Chong and Loehr (1992) it was observed that *Nitrobacter* acclimated to 40 mg/l NH_3 continued to fully nitrify while unacclimated *Nitrobacter* could only tolerate up to 3.5 mg/l NH_3. This is the result of the specific biomass at the Ruabon site treating high ammoniacal nitrogen containing wastewater over a long period, which has resulted in the biomass becoming acclimated to high levels of ammonia that would otherwise be toxic to an unacclimated biomass.

To investigate how specific substances may interfere with the nitrification process the following procedure was adopted. The base synthetic feed matrix was spiked with specific concentrations of the suspected interfering substrate (see Chapter 2 for details). These same substrates were then added in various combinations to assess the additive effects of various inhibitory compounds.

3.10 Effect of thiazoles on nitrifying bacteria in the Vitox system

3.10.1 Compounds used in the study

The following benzothiazoles and derivatives at the concentrations shown were used to investigate their chemical effects on nitrifying organisms: benzothiazole-2-sulphonic acid at 100 mg/l; 2-mercaptobenzothiazole at 25 and 50 mg/l; benzothiazole at 25 and 50 mg/l; and 2-hydroxybenzothiazole at 25 and 50 mg/l. Following inoculation of the biomass with the individual substrates at the two concentration levels, the substrates where then fed to the biomass in various combinations (Table 3.4).

Each experimental run lasted seven days during which the concentration of ammonia (NH_3) was monitored each day at the same time (9 am). The analysis for ammonia was carried out on a 24 hour composite sample of the effluent from the system.

Table 3.4 Matrix for experimental studies.

Concentration mg/l				
BTSA	MBT	BTH	BTOH	NH_3
100	25	0	0	75
100	50	0	0	75
100	25	25	0	75
100	25	50	0	75
100	25	25	25	75
100	25	25	50	75

3.10.2 Results and discussion of chemical effects on nitrification

Contrary to the findings of some workers in the field, it was found that the biomass inoculated with the various benzothiazoles continued to fully nitrify in all cases. At one point in the investigation, when all benzothiazoles and derivatives were being fed to the biomass, a slight increase in the normal level of residual ammonia was observed. This was a very small increase and only occurred over one 24 hour period. Normal residual ammonia levels for this biological reactor are in the range 0.2–0.38 mg NH_3/l; this value rose to 0.58 mg/l NH_3, but within 24 hours was back to normal levels for this reactor.

As initially stated, there has been extensive research done in the area of nitrification and inhibition of nitrification. Most of this work has been looking at the specific mechanisms occurring during the nitrification processes, some have looked at specific substrates and toxicity effects while others have determined rate constants.

The findings of this work are both supportive and contradictory of the literature in that pH is found to have a major impact on the nitrification process. The particular biomass developed at Ruabon has a very high tolerance for ammonia NH_3 at concentrations as high as 75 mg/l NH_3 in the incoming feed. This is in contradiction to the findings of other workers in the field, (Anthonisen *et al.*, 1976; Abeling and Seyfried, 1992) who claim levels in excess of 3–15 mg/l NH_3 are inhibitory to *Nitrobacter*. Contrary to these findings and in support of the results obtained in the present study was work carried out by Horan *et al.* (1997) using granular activated carbon-biological fluidised-bed reactors for the treatment of landfill leachates containing high levels of ammonia which demonstrated that

ammonia levels as high as 220 to 800 mg/l could be treated with a removal efficiency of between 70 and 90% of the incoming ammonia load.

However, the results from the study of benzothiazole effects on nitrification are in stark contrast to previous findings by Tomlinson *et al.* (1966) who observed that 3 mg/l MBT exerted a 75% inhibition on the ammonia oxidation step. Benzothiazole (BTH) was found by Walker (1989) to inhibit activated sludge at a concentration of 650 mg/l, whereas Knapp *et al.* (1982) found that 7 mg/l and 54 mg/l BTH caused a 50% and 100% inhibition respectively of the ammonia oxidation step.

One obvious explanation for the wide differences in findings by the various workers studying nitrification would be that of biological acclimation. This would certainly be the case with the biomass at the Flexsys Ruabon treatment plant where this facility has a long history of treating benzothiazoles and derivatives at elevated concentrations as high as 50 mg/l in the incoming feed. Previous studies on inhibition of the nitrification process have been done in the presence of inhibitors at a given concentration within the biological reactor. This study has looked at how the inhibitory compound behaves in a system which is efficiently degrading all of the thiazoles so their concentration in the biological reactor is effectively zero and hence they have no inhibitory effect – irrespective of their concentration in the feed.

Inhibition is not quite the same as toxicity. Inhibition affects the growth rate constant and the assimilation rate of the substrate but does not necessarily kill the organisms. The onset of inhibition occurs once the substrate concentration reaches an inhibition threshold (illustrated in Figure 3.6). The concentration range over which this inhibition effect is exerted can be quite wide as shown in the graph. Take away the source of inhibition and the organisms should recover.

The above experiments have investigated how the ammoniacal nitrogen load of an incoming feed can affect the nitrifying ability of the specific biomass at the Ruabon treatment works. They illustrate how the laboratory bioreactors used in this investigation model the full-scale treatment plant more closely than can simple static models.

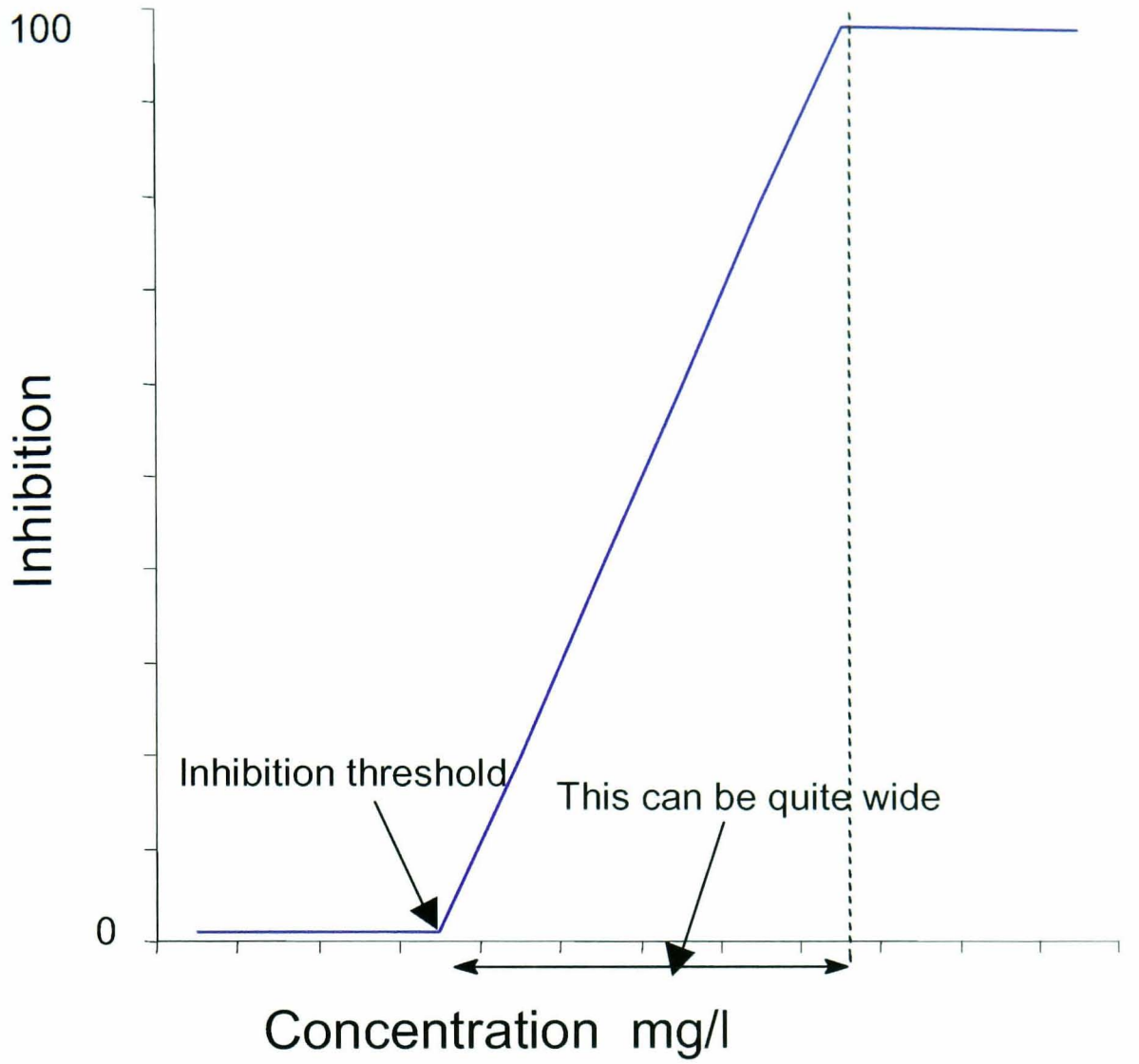


Figure 3.6 Substrate concentration versus inhibition.

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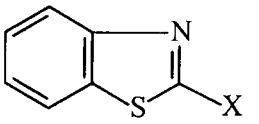
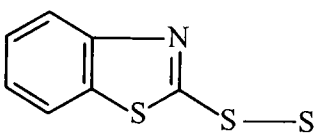
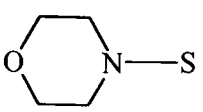
CHAPTER 4
BIOLOGICAL DEGRADATION OF THIAZOLIC COMPOUNDS

4.1 Introduction

Benzothiazoles constitute an industrially important class of heterocyclic compounds. They find use in many industrial applications such as vulcanisation accelerators in the manufacture of rubber *e.g.* 2-mercaptobenzothiazole (MBT) (Santodonato *et al.*, 1976). In the vulcanisation process they catalyse the formation of sulphide linkages between unsaturated elastomeric polymers in order to obtain a flexible and elastic crosslinked material (Wolfe, 1971). They are also used as corrosion inhibitors in antifreeze formulations. 2-Aminobenzothiazole (2ABT) is used in the manufacture of some disperse azo dyes. Structures and names of a range of benzothiazoles are given in Table 4.1.

Table 4.1 Various benzothiazoles and abbreviations (see Appendix 4A for full structures).

General formula 

X	Compound name (abbreviation)
H	benzothiazole (BTH)
CH_3	2-methylbenzothiazole (MeBTH)
SH	2-mercaptobenzothiazole (MBT)
OH	2-hydroxybenzothiazole (BTOH)
NH_2	2-aminobenzothiazole (2ABT)
SCH_3	2-methylmercaptobenzothiazole (MeMBT)
	bis(2-benzothiazolyl) disulphide (MBTS) (also called 2,2′-dithiobisbenzothiazole)
$SOCH_3$	2-methylsulphenylbenzothiazole (MSOBTH)
SO_2CH_3	2-methylsulphinylbenzothiazole (MSO_2BTH)
	2-(4-morpholinothio)benzothiazole (MBS)
SO_3H	benzothiazole-2-sulphonic acid (BTSA)

Although there is an extensive literature on the production and use of benzothiazoles, there are very few reports on their environmental impact and removal from aquatic or terrestrial environments by microflora. Many benzothiazoles have been reported in aquatic systems, *e.g.* river water (Burnham *et al.*, 1973) and drinking water (Coleman *et al.*, 1980). One of the main routes for the entry of benzothiazoles into the environment is via the manufacture and use of MBT and MBT-based rubber additives (Jungclaus *et al.*, 1976). The occurrence of benzothiazole in river water has been proposed as an indicator of the presence of road run-off water in rivers (Spies *et al.*, 1987). The industrial manufacture of benzothiazoles gives rise to wastewaters containing a family of benzothiazoles and intermediates, the bulk of which must be removed prior to discharge into the environment.

Research into the biodegradation of benzothiazoles has been carried out by a number of groups. Biodegradation studies on benzothiazole-2-sulphonic acid (BTSA) were carried out by Mainprize *et al.* (1976) and on 2-hydroxybenzothiazole (BTOH) by De Vos *et al.* (1993a). 2-Mercaptobenzothiazole (MBT) was studied by Chudoba *et al.* (1977) and by De Wever and Verachtert (1994). Chudoba *et al.* suggested that MBT may be recalcitrant to biodegradation. Although it has not been reported that any microbes capable of using MBT as a growth substrate (for carbon and energy) exist, information published by Mainprize *et al.* (1976) and by Gaja (1996) suggests that it may be partially metabolised by microbes which have been acclimated to other benzothiazoles as a carbon source.

MBT has been shown to interfere with nitrification processes and to exhibit biocidal effects (Tomlinson *et al.,* 1966; Czechowski and Rossmore, 1981; Knapp *et al,* 1982). De Vos *et al.* (1993b) demonstrated in laboratory experiments that MBT appeared to be the main component responsible for toxic effects. Mainprize *et al.* (1976), De Vos *et al.* (1993b), and Tomlinson *et al.* (1966) have all studied the behaviour of benzothiazoles in activated sludge systems. The findings of these studies were that BTH and BTOH were treatable whereas MBT and BTSA were not. At the high concentrations used, these compounds became toxic and inhibited biological activity in the order MBT > BTH > BTOH. The BTSA tended to be unaffected and not to exhibit any toxic or inhibition effects towards the biomass.

4.2 Methodology

4.2.1 Chemicals

Pure 2-phenylbenzothiazole, 2-hydroxybenzothiazole and 3-(2-benzothiazothio)-1-propane sulphonic acid sodium salt were obtained from the Aldrich Chemical Company, Poole, Dorset, UK. BTSA was synthesised by Menai Organics Ltd., Bangor, North Wales, UK. All other compounds used in the study were kindly provided by Flexsys Rubber Chemicals.

Dichloromethane, acetonitrile (far UV grade), and cetyltrimethylammonium bromide (CTAB) were obtained through Metlab Supplies Ltd., Hawarden, North Wales, UK. Solid phase extraction cartridges (SPE) manufactured by International Sorbent Technologies Inc, USA were supplied through Jones Chromatography, South Wales, UK.

4.2.2 Culture medium

The biomass used to investigate the biological breakdown of benzothiazoles was obtained from a wastewater treatment plant at the Flexsys Ruabon site with a long history of treating thiazole-containing wastewaters. In one of the laboratory reactors the biomass was pre-treated as described in Chapter 2 Section 2.1.3 prior to inoculation with benzothiazoles.

The prepared culture was fed the synthetic base feed matrix as detailed in Chapter 2, into which the various benzothiazoles were spiked at 50, 75 or 100 mg/l and this composite mixture was fed to the biomass over a ten day period at a rate of 3.5 cm^3/min. Effluent samples were collected continuously during the ten days in discrete 24 hour periods. The collected effluent was thoroughly mixed and a one litre aliquot withdrawn which was filtered through a Whatman No 1 filter paper to remove any solids carried over.

4.2.3 Analytical

All samples were subjected to both liquid–liquid extraction (LLE) (Chapter 2, Section 2.3.1) and solid phase extraction (SPE) (Chapter 2, Section 2.3.2) to isolate analytes of interest.

4.2.4 HPLC analysis

HPLC analyses were performed using a Varian HPLC system; see Chapter 2 for full analytical details for each method used.

4.2.5 GCMS analysis

GCMS analyses were carried out on a Varian gas chromatograph model 3400 CX fitted with a Saturn 3 ion trap mass selective detector. For analytical conditions and instrument configuration see Chapter 2, Section 2.2.

4.2.6 Biodegradation studies

The results for each experiment are presented as discrete sections with a short discussion of results concluding that section. A detailed concluding section then draws all the results together and discusses the subject of thiazole breakdown in relation to the specific bacteria used in the laboratory biological reactors.

4.3 Single substrate biodegradation studies

4.3.1 Benzothiazole

The reactor R3 was inoculated at a rate of 3.5 cm^3/min over a period of ten days with the base matrix feed spiked with benzothiazole (BTH) at 50 mg/l.

When analysis of the effluent collected after the first 24 hour period was carried out, the sample initially appeared to be lacking in BTH and any associated (expected) metabolites. The explanation for this was that initially the incoming substrate was adsorbed onto the surface of the biological floc.

However, by the end of the second day biological activity was observed as indicated by the presence of BTH and a metabolite identified as 2-(methylthio)aniline (Figure 4.1). The chromatogram also showed the presence of phenol, one of the components of the feed to the bioreactor.

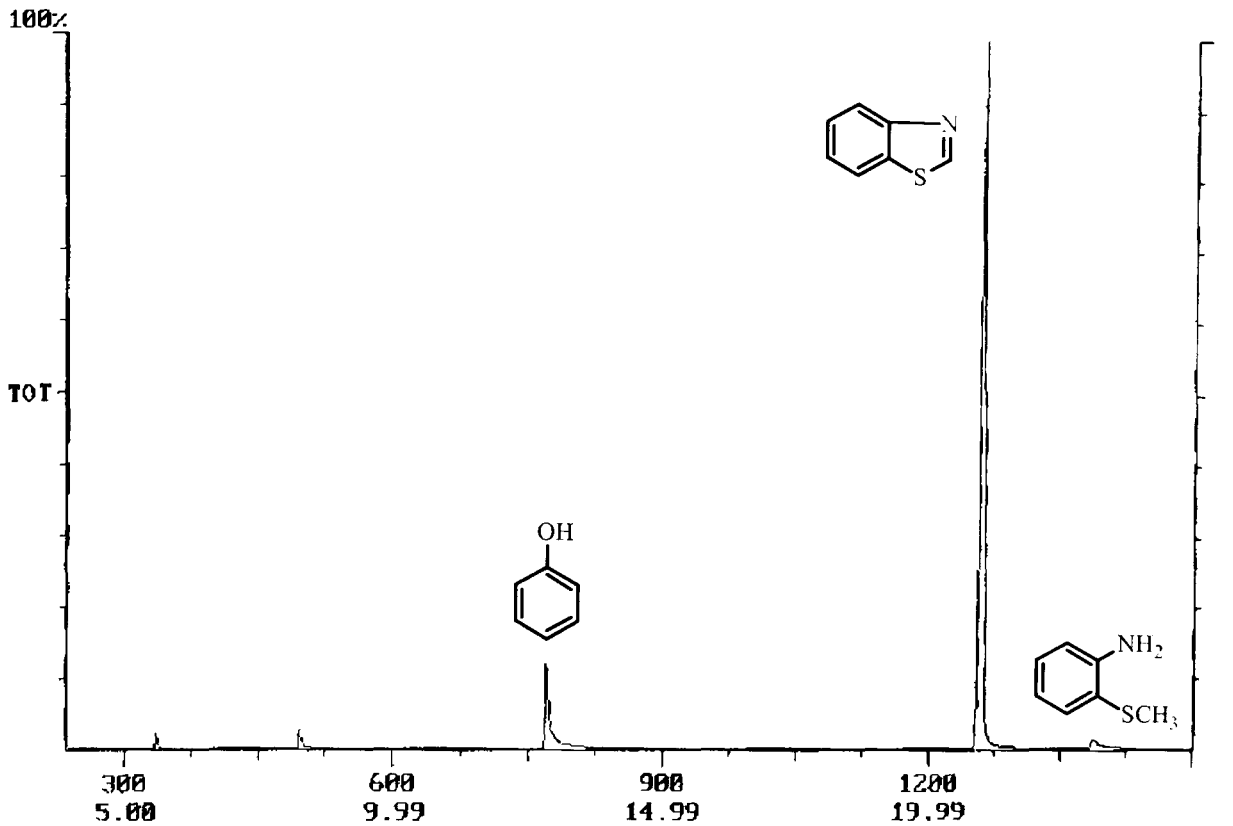


Figure 4.1 BTH GCMS 1.

Analysis of effluent from the bioreactor collected after three days confirmed that the BTH was being biologically degraded with the production of various metabolites. Levels of the metabolite 2-(methylthio)aniline continued to increase with the appearance of three new metabolites. Unfortunately, these metabolites, designated M1, M2 and M3 respectively, could not be identified. The metabolite M1 eluted just at the leading edge of the phenol peak and the metabolite M2 was observed to elute just in front of the BTH peak. Both phenol and 2-(methylthio)aniline levels were seen to increase with time, further confirming that metabolisation of the BTH was proceeding as expected.

By day six of the experiment further metabolites were detected in the effluent as a consequence of the 2-(methylthio)aniline and phenol being metabolised. Furthermore, the three previously unidentified metabolites had been completely removed or were no longer being produced by the biomass. In the chromatogram two new metabolites were apparent, the first, which could not be identified, eluting just after the phenol peak in the chromatogram. The second metabolite was identified as 2-methylaniline (*o*-toluidine).

By day nine of the experiment three further new metabolites were observed in the effluent. Two were identified as 3-methylphenol (*m*-cresol) and 3-methylcatechol respectively but the third could not be identified.

4.3.1.1 Pathway for benzothiazole degradation

On the basis of the detected metabolites the following is proposed as a possible metabolic pathway for the removal of benzothiazole by analogy with related pathways in the literature (Figure 4.2).

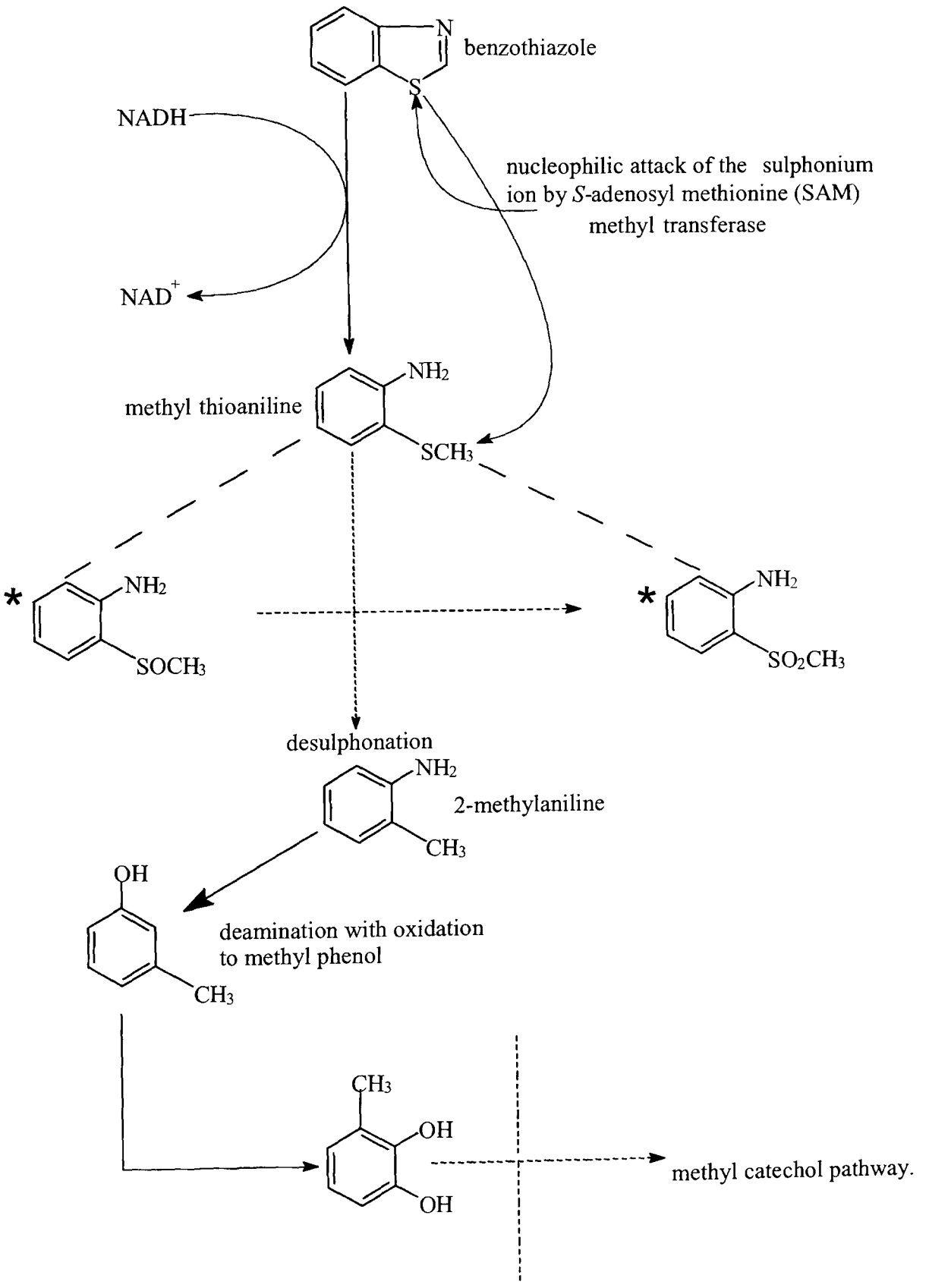


Figure 4.2 Proposed metabolic pathway for benzothiazole biodegradation.

The two compounds marked with an asterisk were not detected but rather inferred through the detection of the metabolite 2-methylaniline. This would facilitate the removal of sulphur from the precursor 2-(methylthio)aniline.

The proposed initial methylation is based on detection of the compound methionine observed during metabolisation of benzothiazole at elevated concentrations from the main wastewater treatment plant. The compound *S*-adenosyl methionine (SAM) is a powerful methylating agent (Drotar *et al.*, 1987) producing methyl radicals that attack the sulphonium ion of the benzothiazole. Figure 4.3 shows the methionine pathway for formation of SAM and the release of methyl radicals.

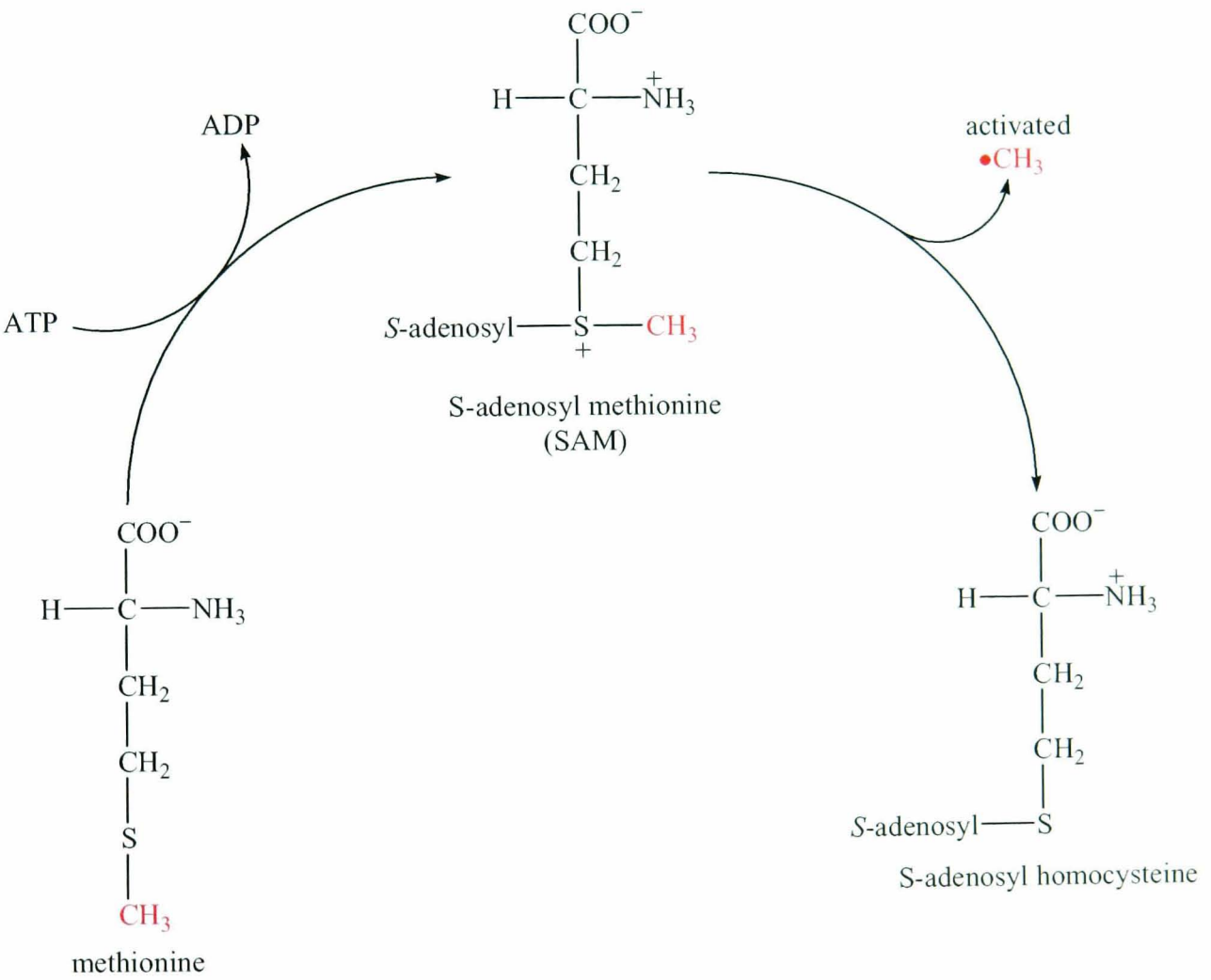


Figure 4.3 Part of the methionine pathway for formation of SAM and the generation of methyl radicals involved in the biomethylation of the sulphonium ion of thiazoles.

4.3.2 2-Hydroxybenzothiazole

2-Hydroxybenzothiazole (BTOH) was spiked at 50 mg/l into the base synthetic feed and fed onto the biomass at a rate of 3.5 cm^3/min in a similar manner to the BTH degradation experiment. Sample collection and preparation were also carried out similarly.

In contrast to BTH, the biomass began metabolising the BTOH within the first 24 hours. This could be due to the more polar nature of BTOH. Analysis of the effluent collected during the initial 24 hours showed this by the presence of the metabolite 2-aminobenzenethiol along with BTOH and three unidentified polar metabolites, P1, P2 and P3 (Figure 4.4).

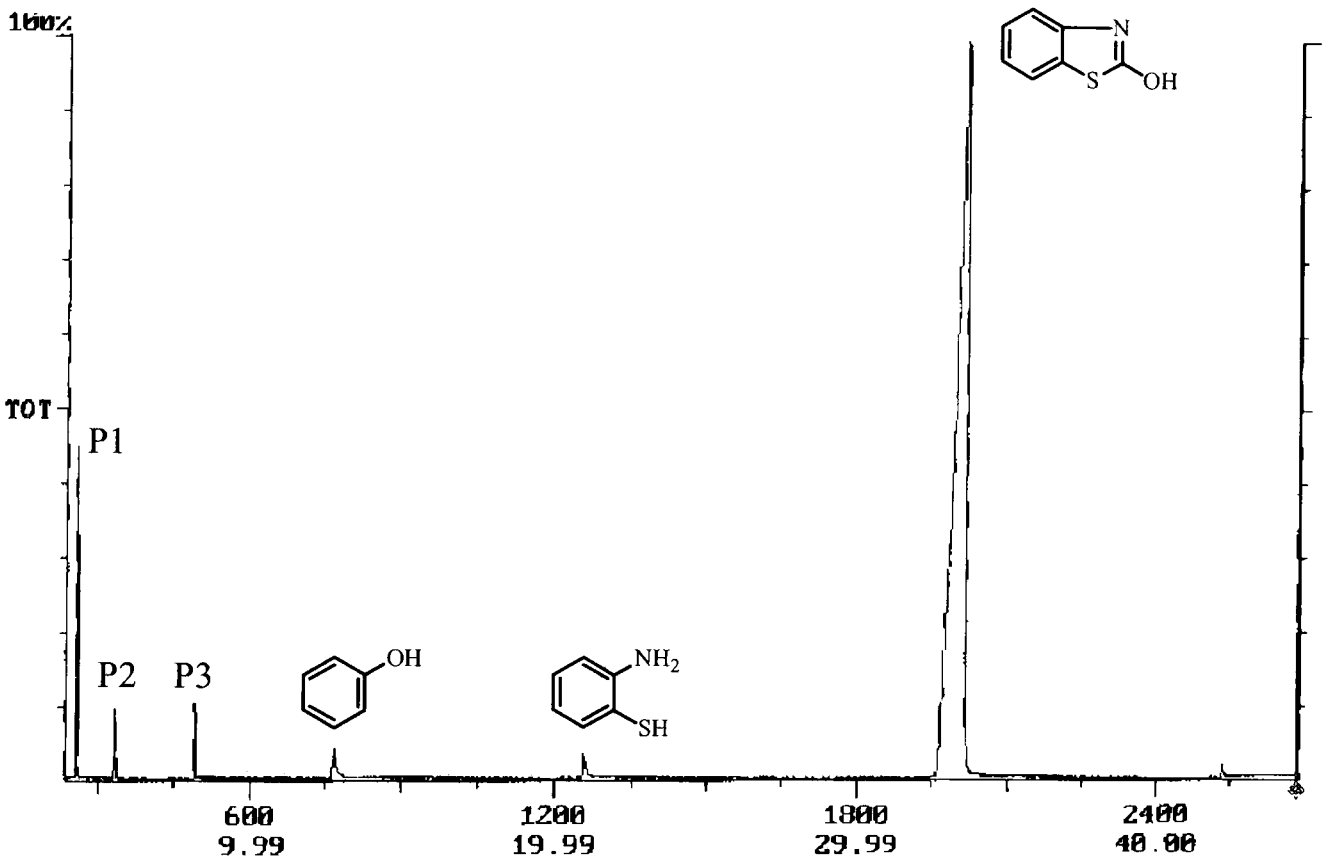


Figure 4.4 BTOH GCMS1. Initial 24 hour effluent sample analysis.

During the following three days a number of additional very minor metabolites were observed none of which were identifiable with any confidence. The mass spectra contained fragments consistent with benzothiazole type fragmentation but not in a recognisable pattern. By the fourth day, the two polar metabolites P2 and P3 and 2-aminobenzenethiol were present at higher levels, with P1 present at a much lower level (Figure 4.5).

Metabolisation of the polar compounds P2 and P3 was observed by day seven of the experiment. The early metabolite 2-aminobenzenethiol was also observed at reduced concentrations by this time. A number of new metabolites were detected, namely catechol, nitrophenol and azobenzene.

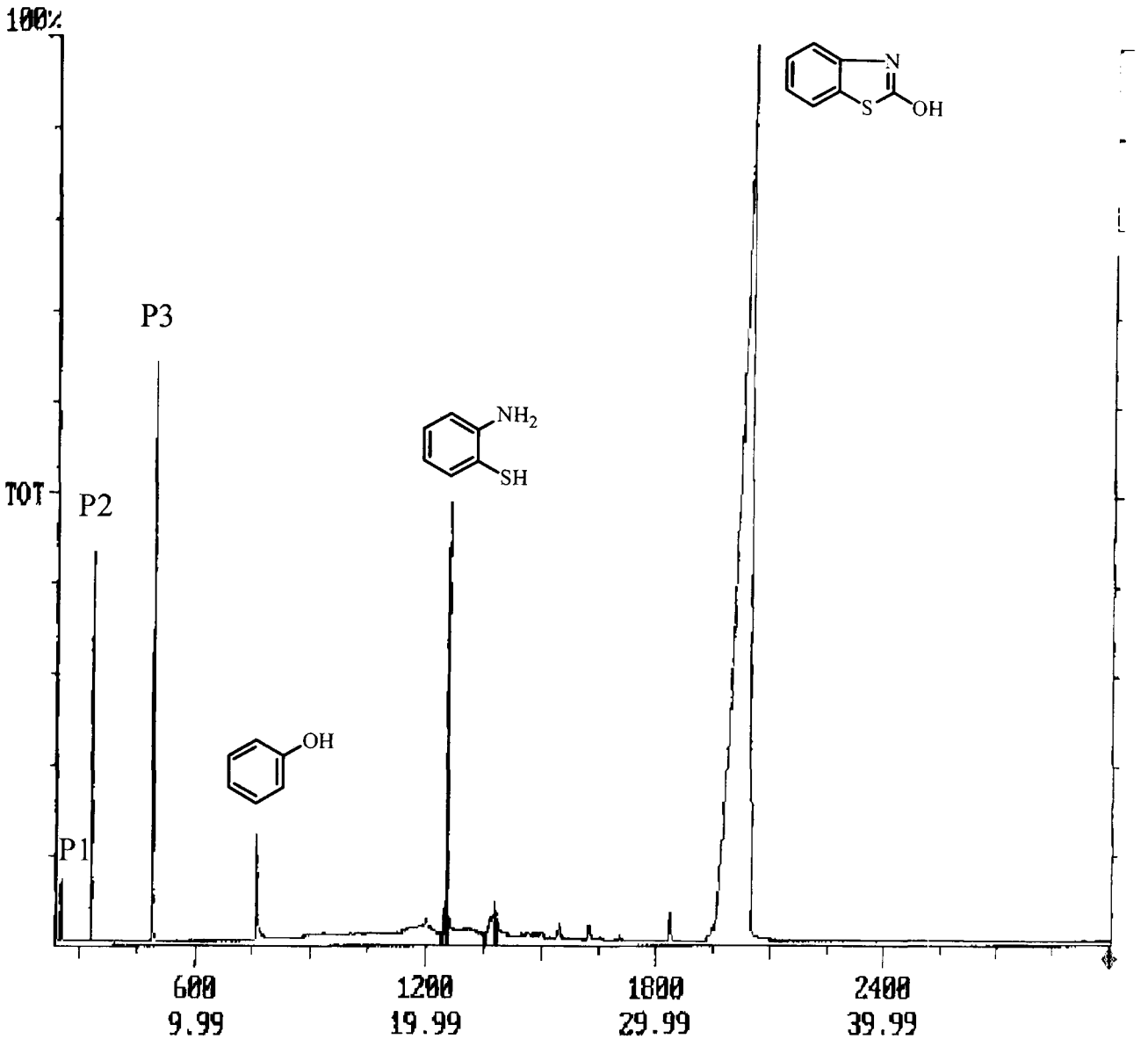


Figure 4.5 BTOH GCMS 2. Effluent collected on day four of experiment.

The presence of nitrophenol is a puzzle as it is difficult to devise a pathway for its formation. It was however transient in its presence, only being present for 24 hours. The various metabolites detected had stabilised in terms of their concentration by day ten, with no new metabolites detected during the last few days of the experiment.

4.3.2.1 Pathway for 2-hydroxybenzothiazole degradation

The scheme shown overleaf (Figure 4.6) is proposed as a possible metabolic pathway for the observed biological breakdown of BTOH based on the limited analytical data collected during the ten day experiment. The thioazobenzene has been included, although not detected in this experiment, as it is observed in the effluent from the main wastewater treatment plant when the feed is known to contain higher than normal levels of BTOH. The thioazobenzene is presumed to be the precursor to the metabolite azobenzene, which is detected in the effluent from bioreactor R3 when exposed to BTOH.

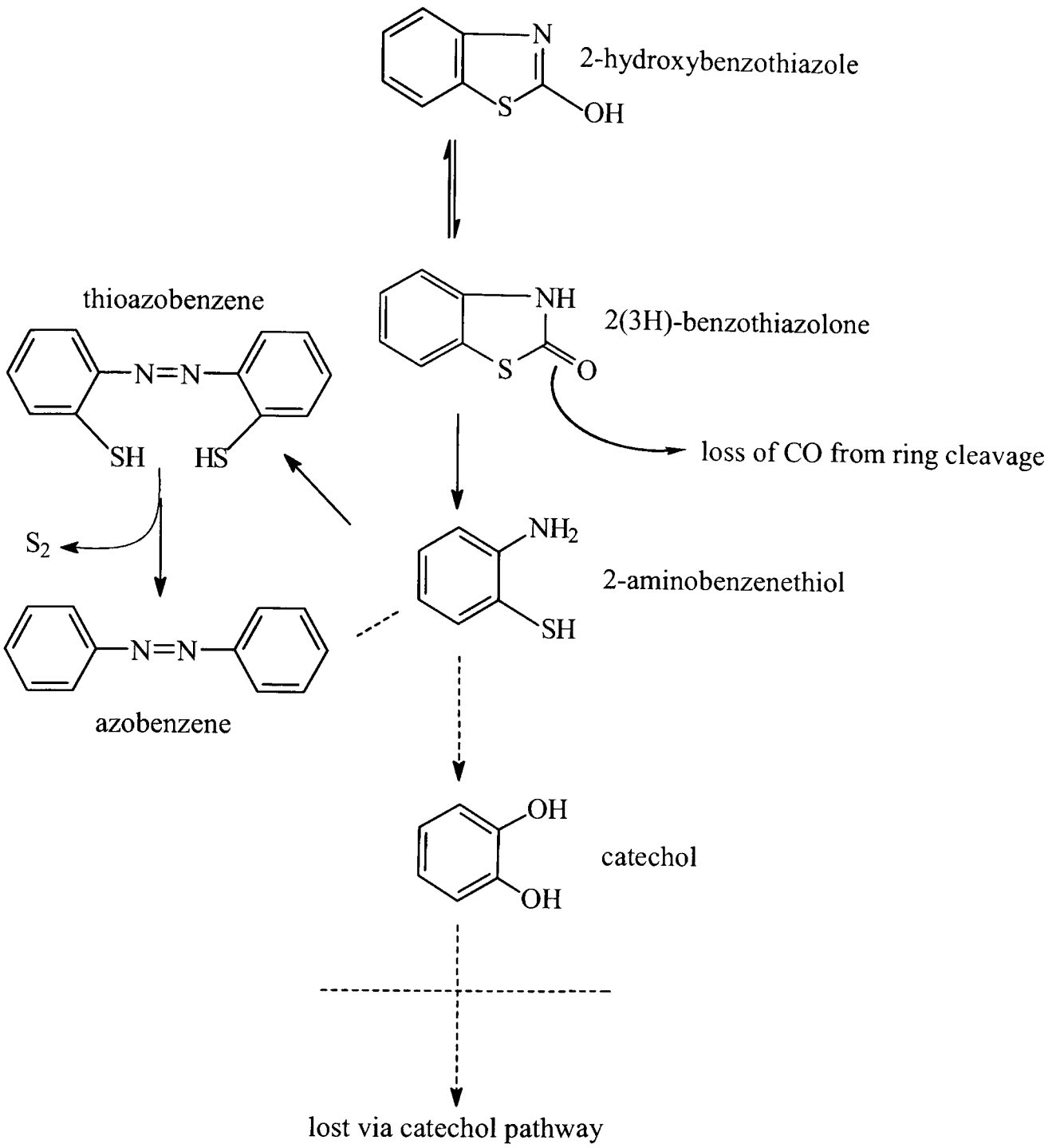
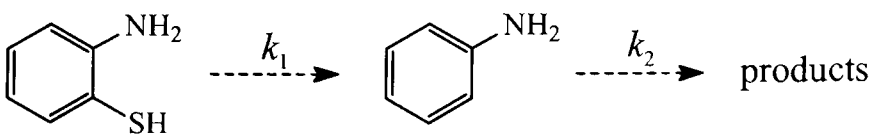


Figure 4.6 Proposed metabolic pathway for 2-hydroxybenzothiazole biodegradation.

The apparent lack of aniline in the effluent is surprising, as it is an expected metabolite of 2-aminobenzenethiol or could be formed from azobenzene, both themselves observed. The absence of aniline does however support the pathway to thioazobenzene and hence to azobenzene, which appears to be a dead end metabolite. A further possible explanation for the fact that aniline is not detected is kinetic control of aniline at low level, with the rate constant for its removal being very much larger than that for its formation.



$$k_2 >>> k_1$$

Simply put, the aniline may have too short a lifetime in the system to be detected.

4.3.3 2-Aminobenzenethiol

To investigate further the fate of 2-aminobenzenethiol (2ABT) in the BTOH pathway, an experiment was conducted in which 75 mg/l of 2-aminobenzenethiol was spiked to the base synthetic feed matrix. This high concentration was chosen in order to maximise the metabolite production and detection. The experiment was run using similar conditions as for the thiazole studies, but was restricted to a seven day period.

4.3.3.1 Results for 2-aminobenzenethiol degradation

Initially the addition of 2-aminobenzenethiol at 75 mg/l had a severe impact on the phenol removal rate, as shown by the analysis of effluent collected during the first 24 hours of the experiment (Figure 4.7). In this chromatogram a large phenol peak is observed, much larger than observed when benzothiazoles are added to the feed. Two unidentified minor metabolites were also detected.

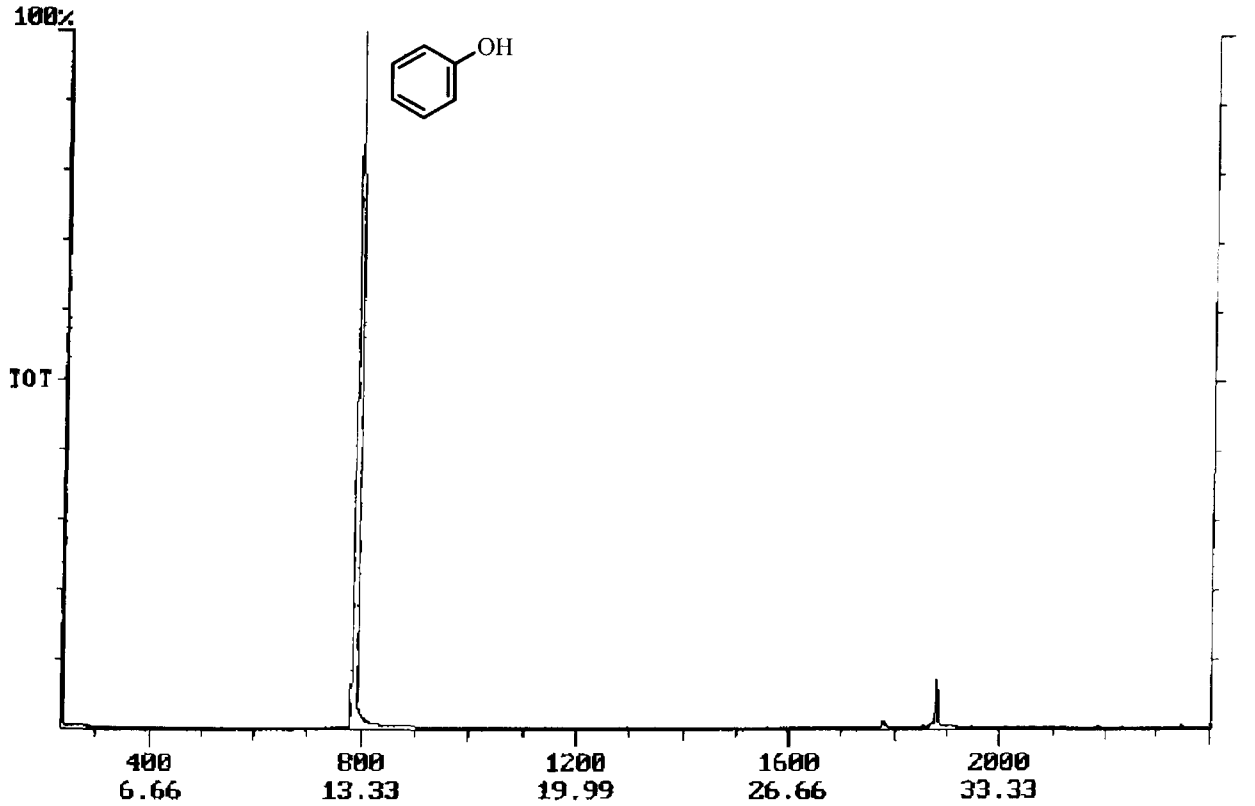


Figure 4.7 AMT GCMS 1. The first 24 hours of 2-aminobenzenethiol breakdown.

Over the next six days, as well as 2-aminobenzenethiol, the effluent was found to contain increasing levels of the following metabolites: BTH, 2-(methylthio)aniline, thioazobenzene and azobenzene (Figure 4.8).

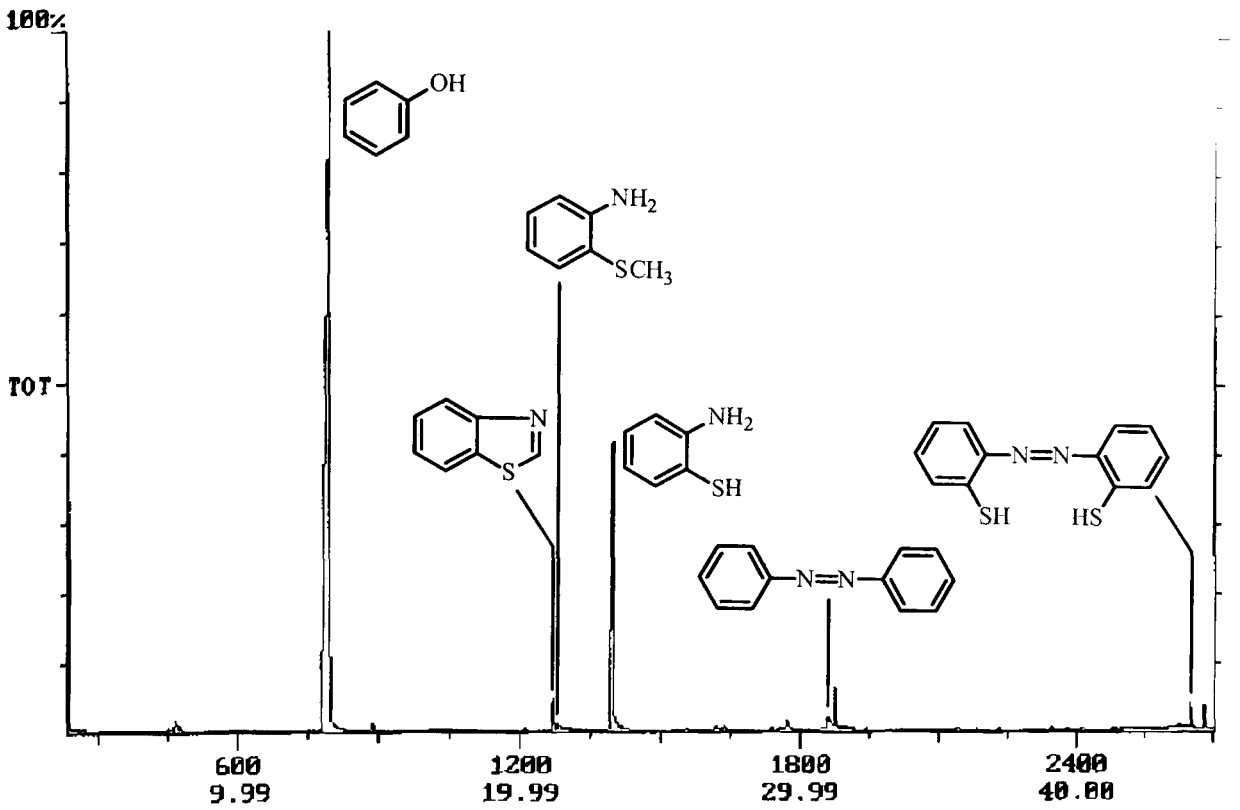


Figure 4.8 AMT GCMS 4. Day seven of 2-aminobenzenethiol breakdown.

4.3.3.2 Fate of 2-aminobenzenethiol

It would appear that initially the 2-aminobenzenethiol inhibits the carbonaceous removal of phenol as evidenced by the large phenol peak observed in chromatogram AMT GCMS 1 (Figure 4.7). The 2-aminobenzenethiol then breaks down via an initial methylation step to yield very low levels of the metabolite 2-(methylthio)aniline, which is in turn transformed via ring closure into BTH. This is the reverse of the findings of the BTH metabolisation study in that during BTH breakdown 2-(methylthio)aniline is formed as a metabolite. It is also confirmed that 2-aminobenzenethiol is a precursor to both thioazobenzene and azobenzene as both of these metabolites were detected during the breakdown process.

The lack of 2-methylaniline in this pathway would support the claim for the metabolic route from 2-aminobenzenethiol to 2-(methylthio)aniline to thioazobenzene as a dead-end metabolite. The connection between 2-aminobenzenethiol and catechol is unfortunately still elusive and not proven in the course of the above experiment. The proposed removal/transformation pathway for 2-aminobenzenethiol is summarized in Figure 4.9.

This sequence of events only becomes observable when elevated levels of 2-amino-benzenethiol are present, *i.e. ca* 75 mg/l. When the 2-aminobenzenethiol is generated as a metabolite as part of the BTOH pathway, it is only present at trace levels and any

metabolites generated during the breakdown of the generated 2-aminobenzenethiol are below the detection limit and so are not observed in the effluent during BTOH breakdown.

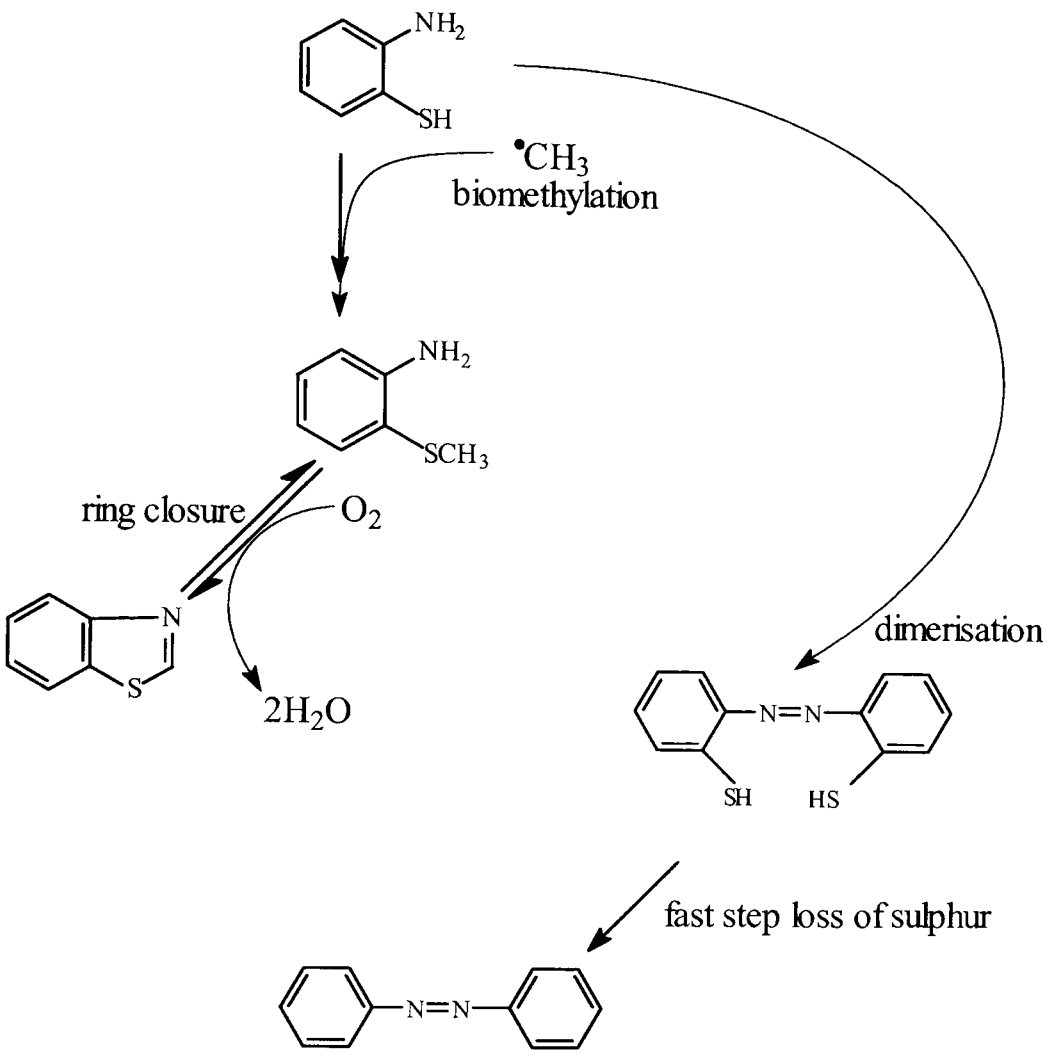


Figure 4.9 Postulated removal/transformation pathway for 2-aminobenzenethiol.

4.3.4 2-mercaptobenzothiazole

2-Mercaptobenzothiazole (MBT) degradation has been studied previously by many workers: Gaja and Knapp (1998) examined the removal of MBT by activated sludge; Fiehn *et al.* (1998) investigated the ozonation of MBT in water and tannery wastewaters; and Brownlee *et al.* (1992) studied the aquatic environmental chemistry of various benzothiazoles. All of these studies were carried out using batch fill and draw type methodology and not as continuously fed batch experiments.

In the present study, the biodegradation of MBT was examined under continuous feed batch conditions: the base synthetic feed was spiked with MBT at 50 mg/l and fed onto the bioreactor R3 at a rate of 3.5 cm^3/min. Analysis (using HPLC Method 1) of effluent

collected during the first 24 hours of the experiment indicated that MBT was being broken down with the production of a few unidentified polar metabolites along with residual untreated MBT (Figure 4.10).

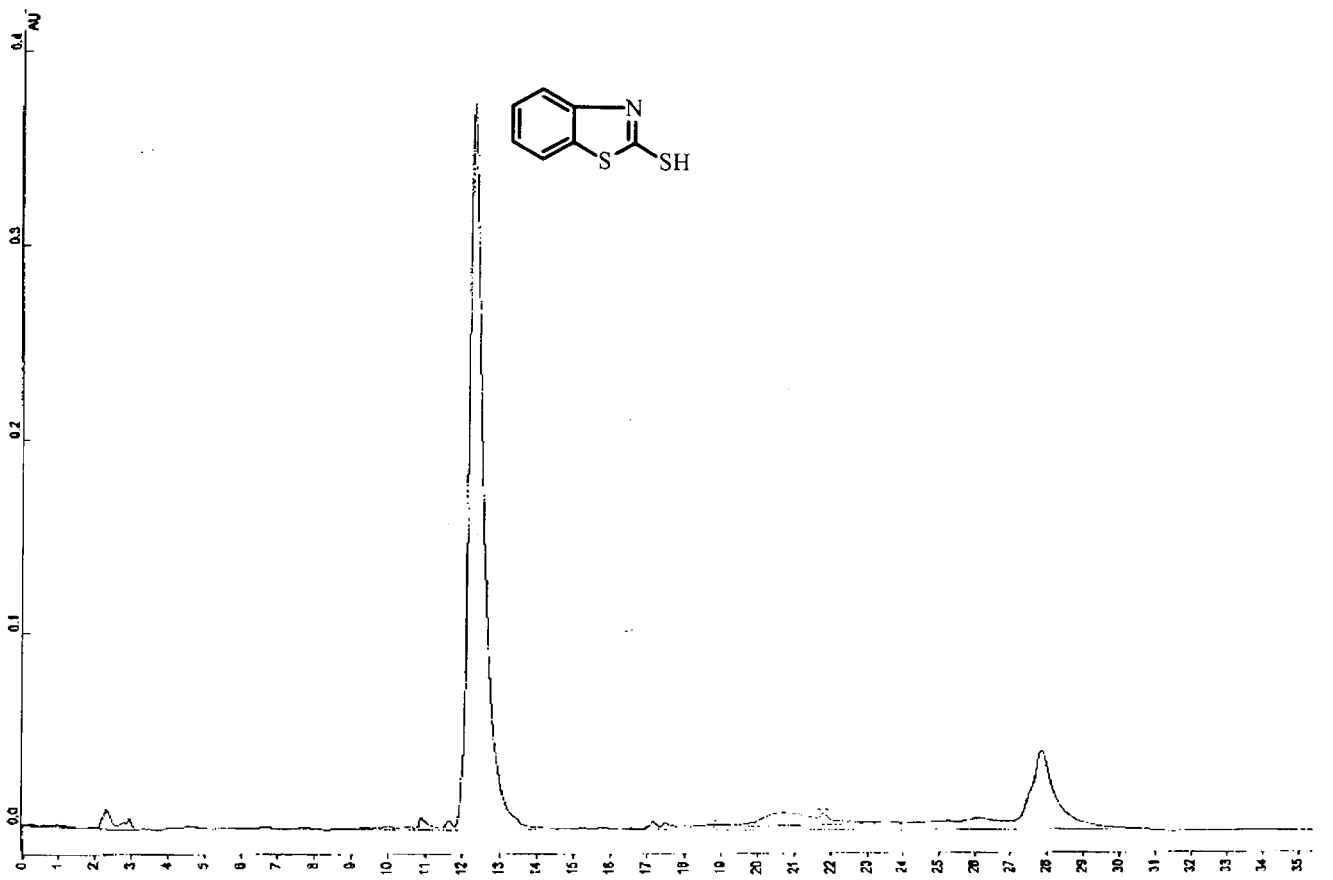


Figure 4.10 MBT HPLC 1. The first 24 hours of MBT metabolisation.

Analysis of the effluent collected after 48 hours showed the presence of MBT, phenol and increasing amounts of other polar metabolites. By day three the effluent was found to contain 2-methylmercaptobenzothiazole (MeMBT) together with phenol and MBT (Figure 4.11). The previously unidentified polar compounds also became more pronounced. By the end of day three the incoming MBT was observed to have an adverse effect on the removal of phenol. Biomethylation was also seen to have taken place with the production of the metabolite MeMBT along with other unidentified intermediates (Figure 4.11).

By the end of day four, various additional metabolites were detected. The previously unidentified metabolite with a retention time of 27.5 mins was identified as butyric acid. The increased concentration gave a better quality spectrum so that assignment by library identification became possible. The new metabolites detected were benzothiazole sulphenic acid (BTSOH) and 2-hydroxybenzothiazole (BTOH). Phenol was still present but at a reduced level while the MeMBT was seen to be increasing in concentration.

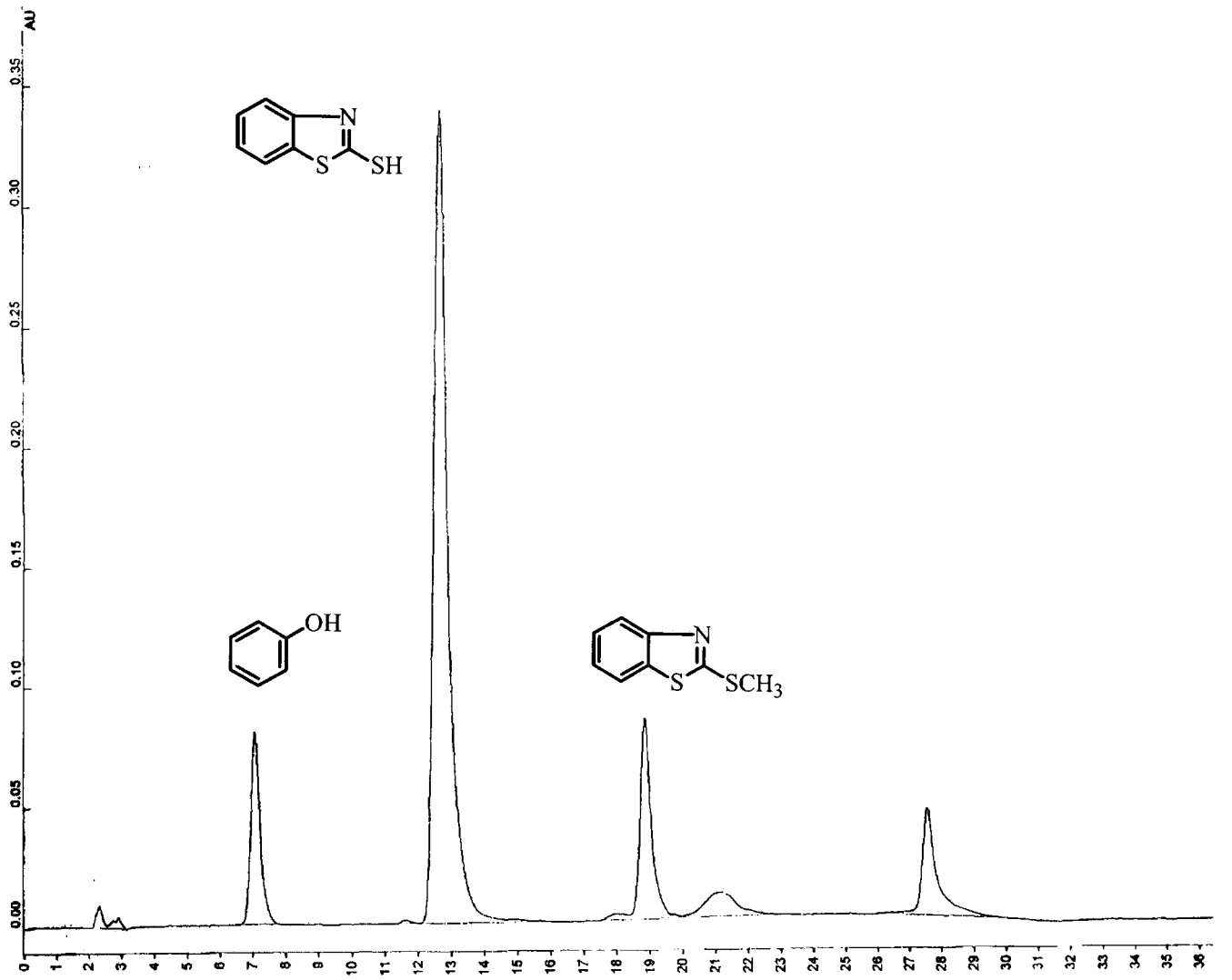


Figure 4.11 MBT HPLC 3. Day three of the MBT metabolisation.

Analysis of the effluent collected on the morning of day six of the experiment showed that the BTSOH levels were slightly elevated along with BTOH. Both phenol and MeMBT levels had fallen slightly. 2-Aminobenzenethiol at significant levels was also observed. Peaks at t_R 21.1 mins were present in chromatograms MBT HPLC 3 and 4, but the spectrum obtained for the peak was not consistent with that for 2-aminobenzenethiol. The peak at t_R 21.1 mins in chromatogram MBT HPLC 5 was of a greater intensity and gave rise to a better quality spectrum from which the assignment could be made (Figure 4.12).

Analysis of effluent from the day seven day indicated that metabolisation was proceeding at a steady rate since no new metabolites were observed and those that were present had not increased to any significant degree. The levels of butyric acid and BTSOH had fallen slightly. By the morning of day nine a new metabolite was detected in the effluent along with a marked reduction in the level of butyric acid and BTSOH levels continuing to fall slowly. The new metabolite eluting before phenol was identified as catechol.

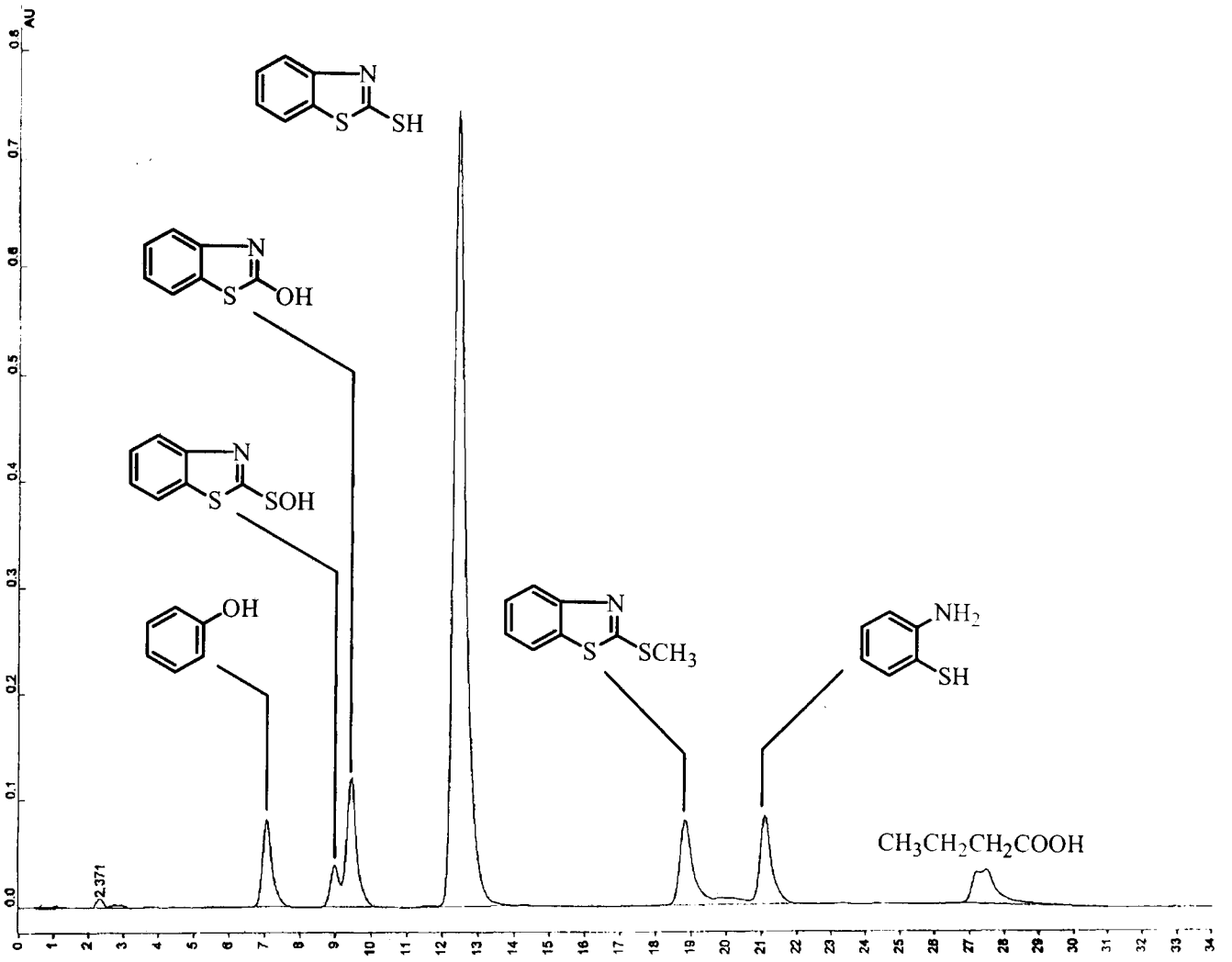


Figure 4.12 MBT HPLC 5. Day five of the MBT metabolisation.

The levels of catechol increased over the final two days of the experiment with no further identifiable metabolites being observed. Both the BTSOH and butyric acid levels increased slightly towards the end of the experiment.

4.3.4.1 Pathway for MBT degradation

HPLC analysis of effluent samples collected each day produced results that allowed a detailed mapping of the biological degradation pathway for MBT by the specific biomass used in this experiment. The results confirmed the findings of other workers in that initial transformation appears to be a biomethylation step. Brownlee *et al.* (1992) also observed biotic methylation of MBT, and Drotar *et al.* (1987) observed methylation of MBT by cell-free extracts of bacteria (*Carynebacterium* sp. and *Pseudomonas* sp.) isolated from soil and water.

In their study, Fiehn *et al.* (1998) found the initial step was loss of sulphur to yield the metabolite benzothiazole (BTH) as a precursor to 2-hydroxybenzothiazole (BTOH) (or its tautomer 2(3H)-benzothiazolone). The results of the current study appear to indicate that

BTH is not an intermediate, but rather that the MeMBT is transformed stepwise to yield both BTOH and the sulphenic acid (BTSOH). At no time was the sulphonic acid metabolite BTSA observed, in contradiction to the findings of Fiehn *et al.* (1998) and De Wever and Verachtert (1994) who observed BTSA formation from MBT. All workers observed incomplete removal of MBT, which is supported by the findings of this study (Figure 4.13).

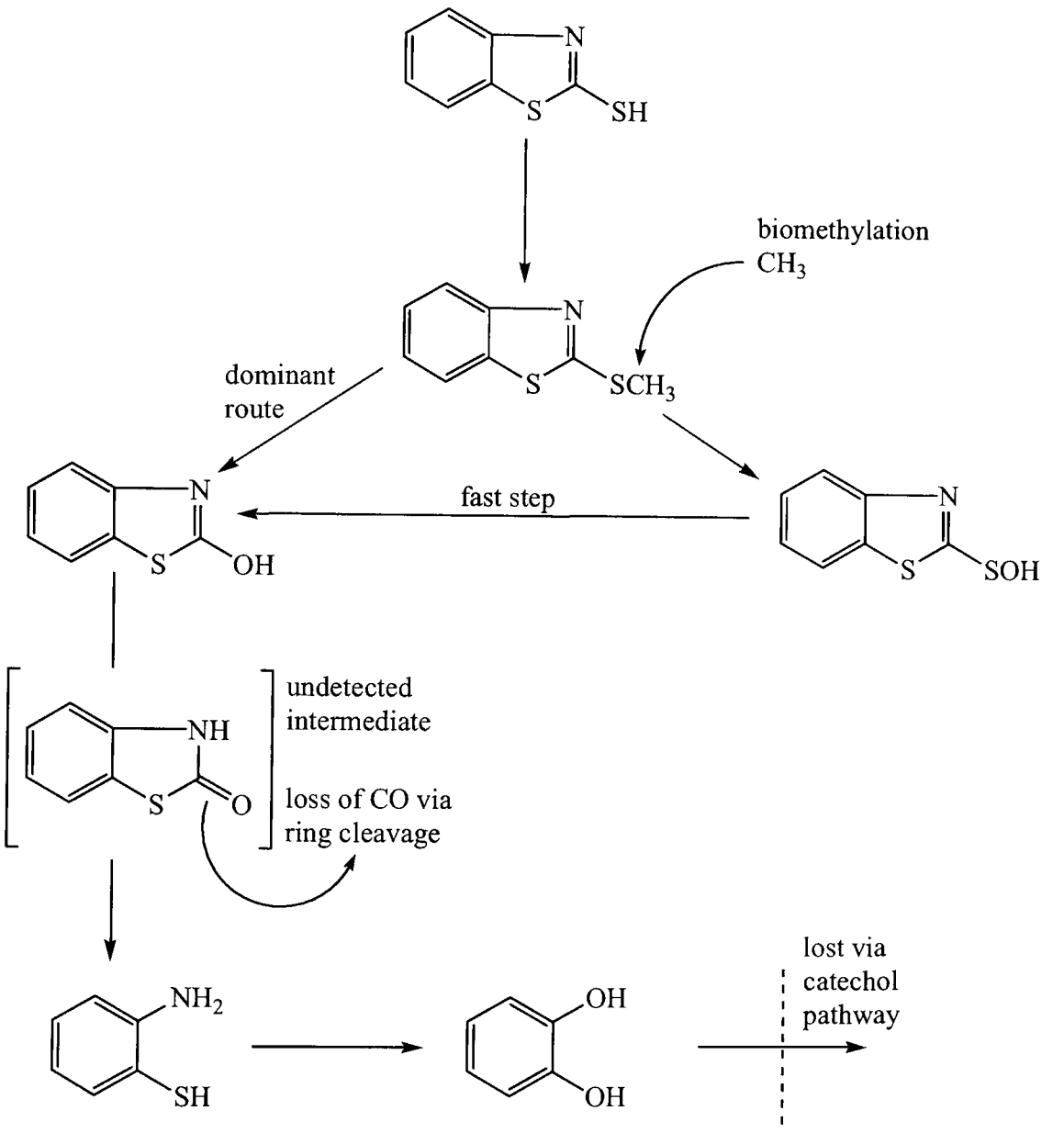


Figure 4.13 Fate pathway for MBT at 50 mg/l.

The presence of 2-aminobenzenethiol confirms the findings of Fiehn *et al.* (1998) who have shown it to be a metabolite along the 2-hydroxybenzothiazole (BTOH) pathway. The 2-aminobenzenethiol probably arises as a consequence of ring cleavage with concomitant loss of 'CO' from the BTOH tautomer 2(3H)-benzothiazolone.

The results obtained for MBT metabolisation at 50 mg/l demonstrated that at this concentration biological activity was not unduly inhibited and that MBT removal

continued at a steady rate as indicated by the production of the observed metabolites over time. However, unlike other workers (De Wever and Verachtert, 1994; Fiehn *et al.*, 1998) who all observed the production of benzothiazole-2-sulphonic acid (BTSA) during MBT metabolisation studies, no BTSA was detected in this work.

As no BTSA was detected during MBT metabolisation at 50 mg/l, a second experiment was run where the MBT concentration was increased to 100 mg/l. All other parameters were kept identical to those used for the study with MBT at 50 mg/l.

4.3.4.2 Results of 100 mg/l MBT metabolisation

Sample collection and preparation along with all analytical conditions were the same as for the MBT 50 mg/l experiment. Analysis (using HPLC Method 1) of the first 24 hour sample clearly indicated that at 100 mg/l MBT, the MBT was oxidised (dimerised) to yield MBTS. At the same time a number of unidentified metabolites were observed with untreated MBT and the residual level of MBT was greater than that observed during metabolisation studies at the 50 mg/l concentration (Figure 4.14).

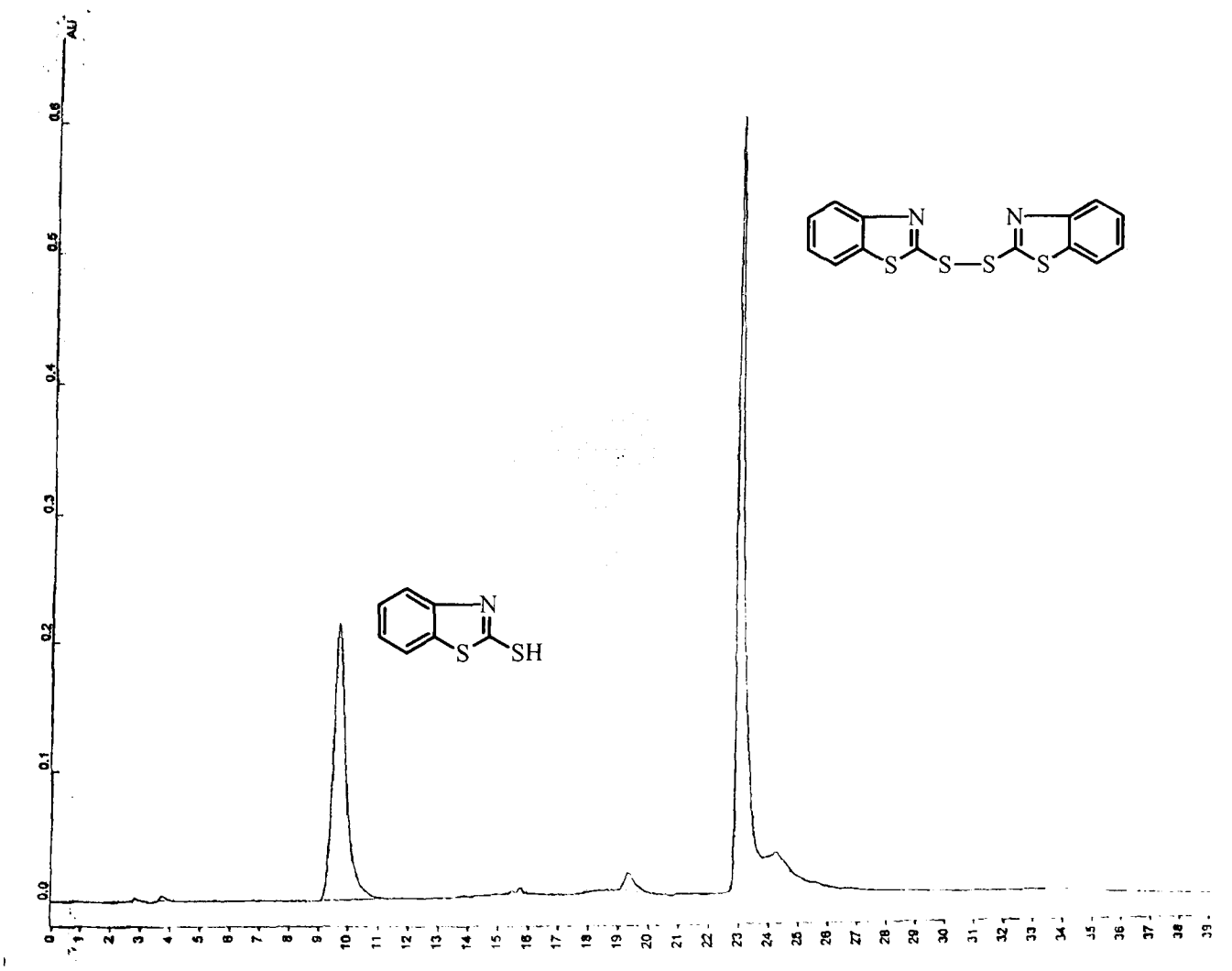


Figure 4.14 MBT HPLC 11. The first 24 hours.

By the end of day two of the experiment, levels of MBTS continued to increase along with various unidentified metabolites, MBT still being present at significant levels. By the end of day three, three new metabolites were detected in the effluent from the reactor. Two of these metabolites were identified as benzothiazole-2-sulphenic acid (BTSOH) and benzothiazole (BTH), with the third unidentified. The MBT and MBTS levels remained constant.

Analysis of effluent collected after four days of MBT metabolism indicated that both the MBT and MBTS levels had increased slightly with a simultaneous increase in BTSOH and production of the metabolite 2-hydroxybenzothiazole (BTOH). Furthermore, the BTSOH appears to have been partially oxidised to benzothiazole-2-sulphinic acid ($BTSO_2H$). The concentration of BTOH and BTSOH is difficult to assess due to the co-elution of these two compounds; furthermore, they have very similar UV spectra (see Figures 4.15 and 4.16 for UV spectra for BTOH and BTSOH respectively). This spectral similarity also makes it difficult to separate these compounds on the basis of their spectra.

By the end of day five of the experiment, levels of MBTS had fallen, with the MBT concentration stabilising. Both $BTSO_2H$ and BTOH levels had increased with the production of a further two metabolites. The two new metabolites were identified as methylbenzothiazole (MeBTH) and methyl mercaptobenzothiazole (MeMBT).

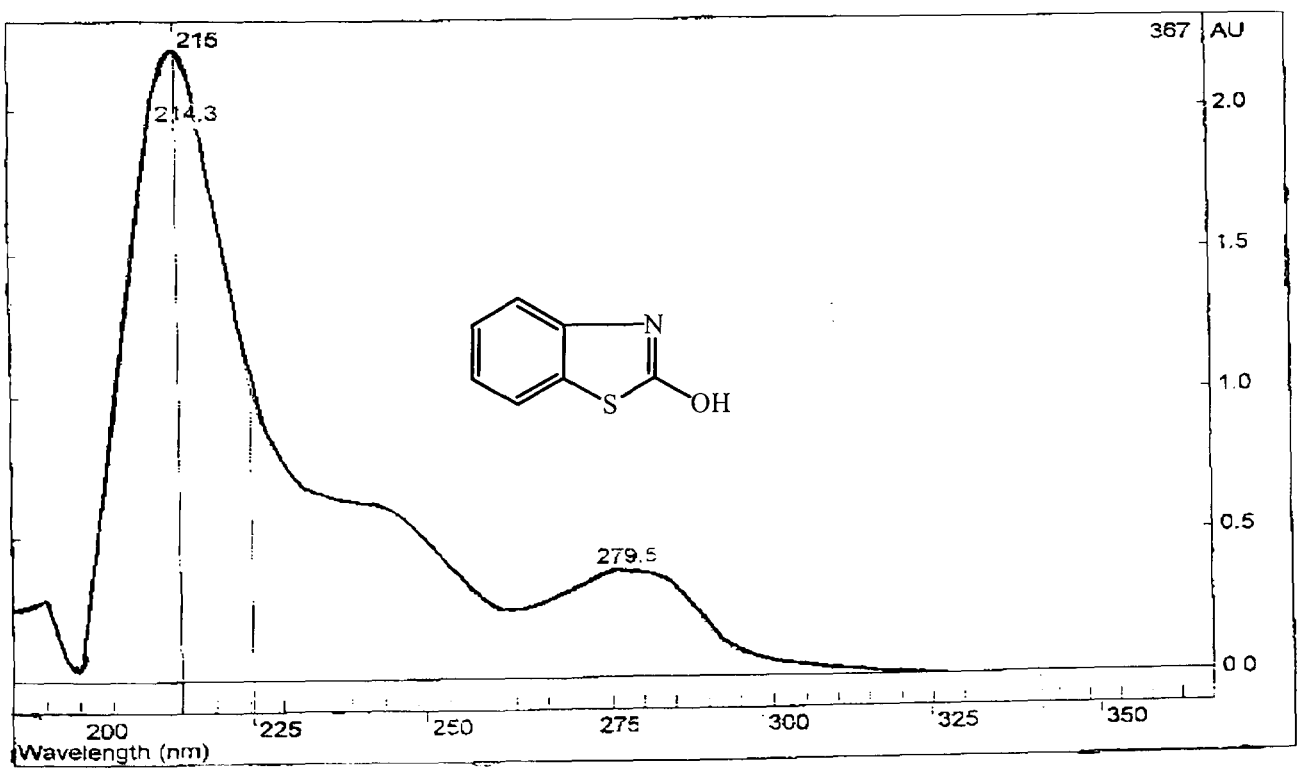


Figure 4.15 UV spectrum of 2-hydroxybenzothiazole (BTOH).

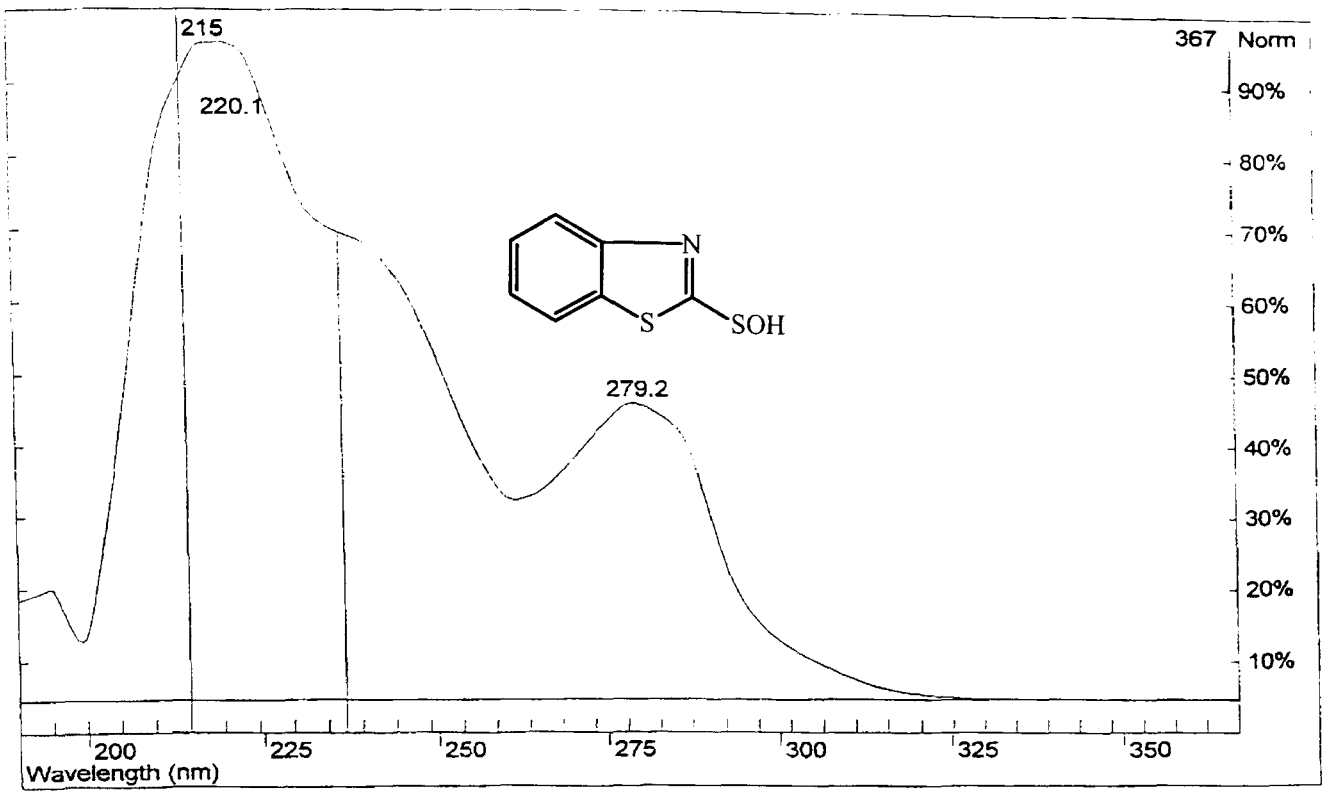


Figure 4.16 UV spectrum of benzothiazole-2-sulphenic acid (BTSOH).

In an attempt to resolve the BTOH/BTSOH co-elution problem, the sample run as MBT HPLC 15 was reanalysed using a different eluent system and gradient profile, but with the same C_{18} Metsphere silica column, using HPLC Method 2. The results of the analysis using the above conditions confirmed that the BTOH/BTSOH peak was principally BTOH with a trace of BTSOH. Furthermore, it was found that the MeBTH was the 5-methylBTH isomer not 2-methylBTH and that BTH was still present at a significant concentration, as was MeMBT (Figure 4.17).

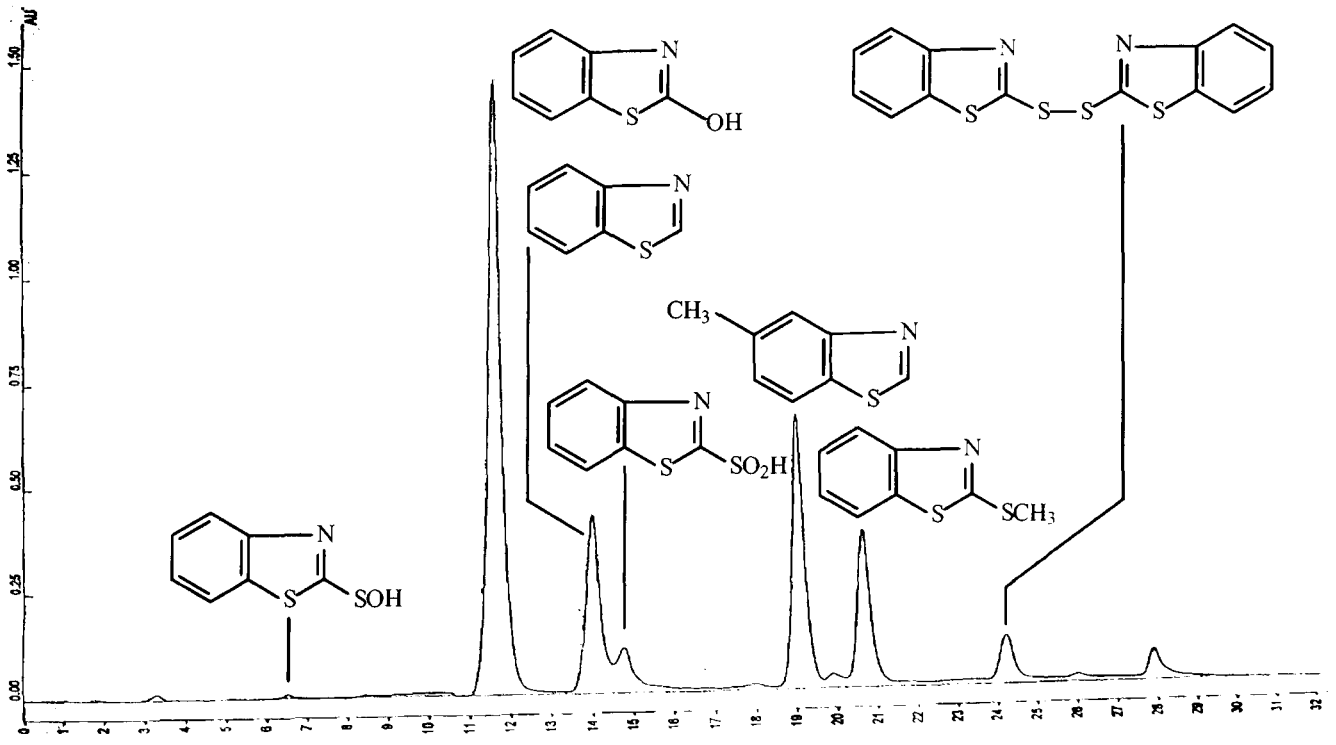


Figure 4.17 MBT HPLC 15b.

Analysis of effluent collected from the bioreactor on the morning of day six of the experiment confirmed that metabolisation was proceeding at a steady rate but with no new metabolites being observed in the effluent. By the end of day seven, *m*-toluidine was detected. However, the new gradient profile (HPLC method 2) had the effect of causing the BTSOH and BTOH to coelute; the same effect was observed with 5-MeBTH and MeMBT (Figure 4.18).

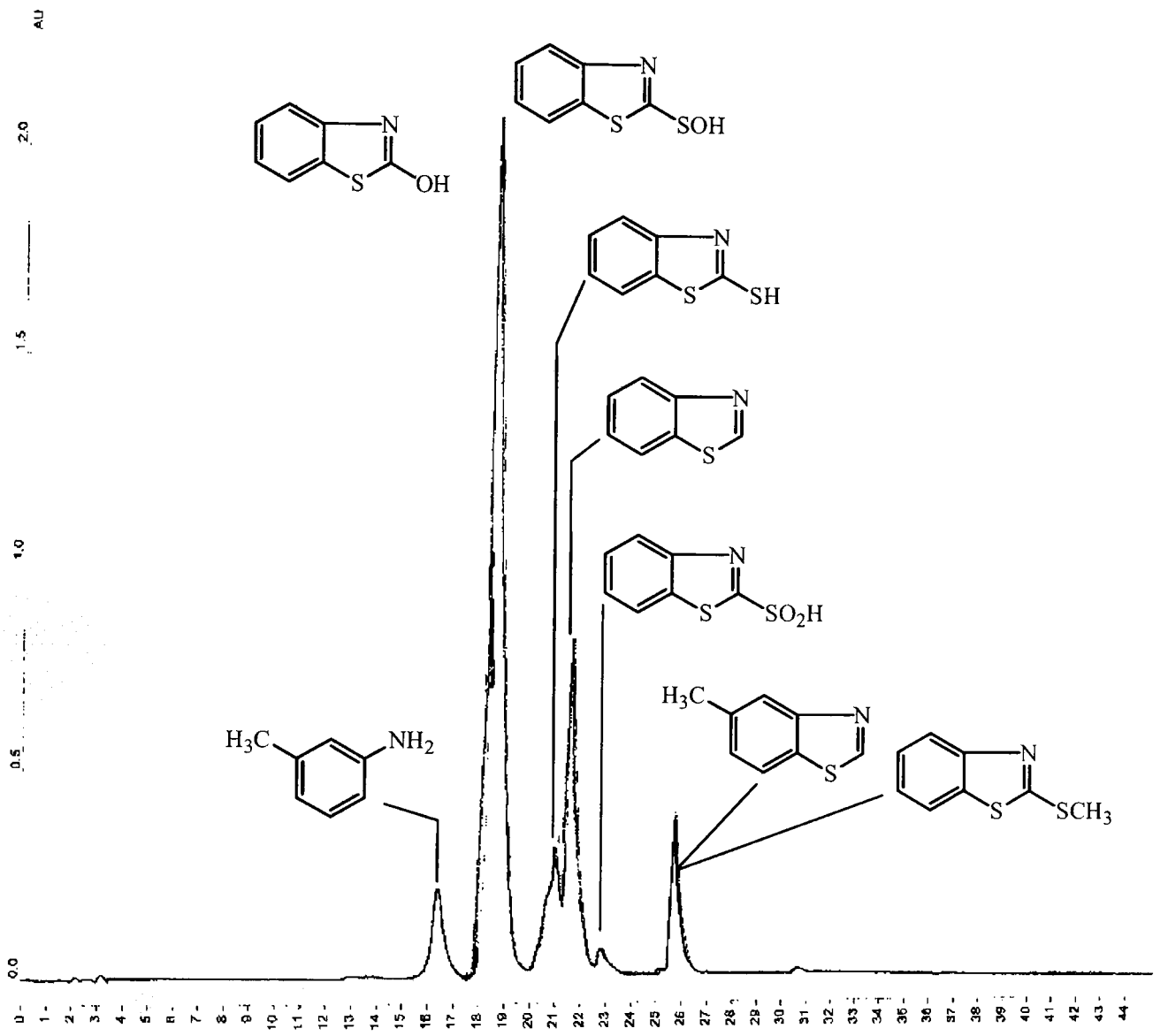


Figure 4.18 MBT HPLC 16. Day seven.

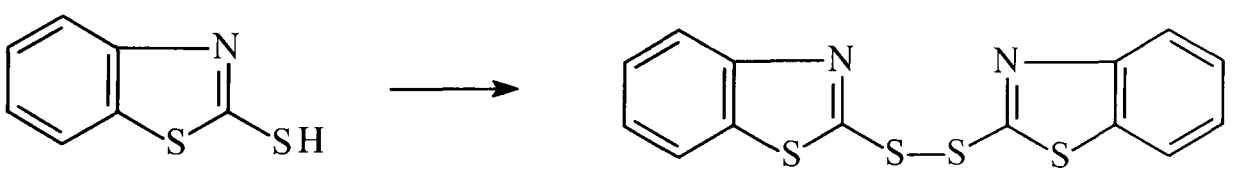
Using the above gradient profile to analyse effluent collected at the end of day eight of the experiment, two further metabolites were detected, 3-methylcatechol and *m*-cresol respectively. The picture did not change by the end of the experiment, with no further metabolites identified.

4.3.4.3 Pathway for 100 mg/l MBT degradation

At low concentrations, *ca* 50 mg/l or less, MBT is removed at a steady rate with the production of a series of metabolites including the methylated species 2-methylmercapto-benzothiazole (MeMBT) along with the sulphenic acid (BTSOH) and 2-hydroxy-benzothiazole (BTOH). It was assumed that the sulphonic acid would feature in the pathway for MBT removal but this was shown not to be the case in contrast to the findings of Fiehn *et al.* (1998). *It should be noted that depending on the degree of acclimation, the absolute levels of MBT will vary, but it is assumed that the mechanisms as proposed by Fiehn* et al. *and that found during this study will still operate.* However, when the MBT concentration in the feed to the bioreactor is increased to 100 mg/l, a different pathway is observed.

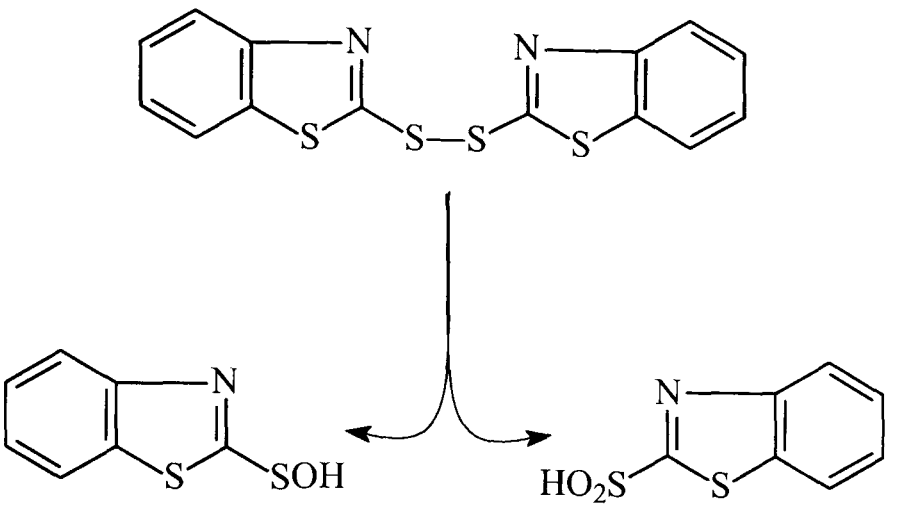
It is known that MBT is toxic to microorganisms at higher concentrations. De Wever and Verachtert (1994) demonstrated that MBT was the main toxic component in thiazolic wastewaters. In the above experiment, MBT at 100 mg/l appears not to exert any significant toxicity towards the biomass used as evidenced by the steady production of metabolites as the MBT is progressively broken down. The formation of MBTS may well be a protective mechanism whereby the biomass transforms the relatively high toxicity substrate MBT into a less toxic substrate, which initially accumulates within the sludge floc prior to biological breakdown. The following pathway is proposed on the basis of analysis of effluent collected during metabolisation studies of MBT at 100 mg/l.

The initial observation was that MBT at 100 mg/l is dimerised (oxidised) to generate the product MBTS.



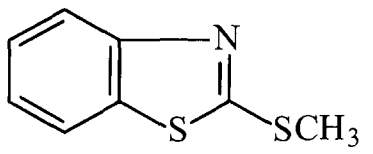
MBT MBTS

This initially accumulates within the sludge floc and is slowly broken down to yield the two metabolites BTSOH and $BTSO_2H$, sulphenic and sulphinic acids respectively.



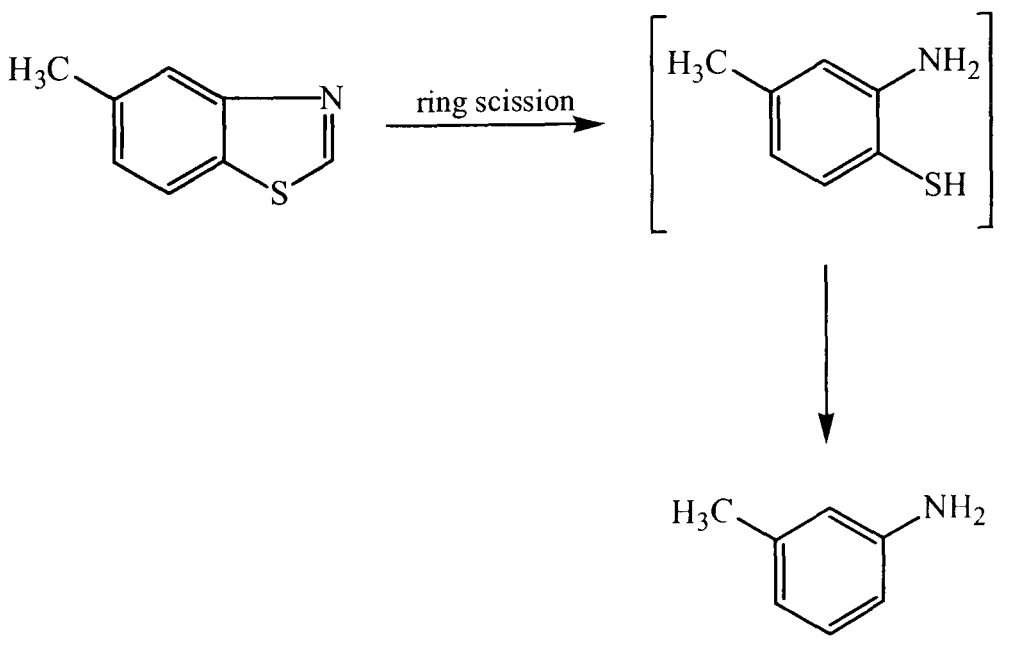
Both of the above compounds are relatively unstable and are rapidly transformed into BTH and BTOH with the liberation of sulphur dioxide ($-SO_2$). The BTH and BTOH are then removed via formation of methylbenzothiazole (MeBTH).

Methylation was also expected to occur at the hetero sulphur atom or even the hetero nitrogen atom; however, it was observed that in this instance methylation occurred at the 5 position on the aromatic ring. Methylation of MBT was also observed but in the normal position to yield 2-methylmercaptobenzothiazole (MeMBT).



2-methylmercaptobenzothiazole

The presence of *m*-toluidine is confirmatory evidence for the 5-MeBTH metabolite and it is possible to infer ring scission with the production of the non-detected intermediate 5-methyl-2-thioaniline.



Under normal conditions, *i.e.* less than 50 mg/l MBT, *o*-toluidine is observed in effluent samples from the main wastewater treatment plant. Deamination of *m*-toluidine followed by hydroxylation gives rise to *m*-cresol and 3-methylcatechol respectively.

The full proposed pathway for MBT biodegradation at 100 mg/l is given in Figure 4.19.

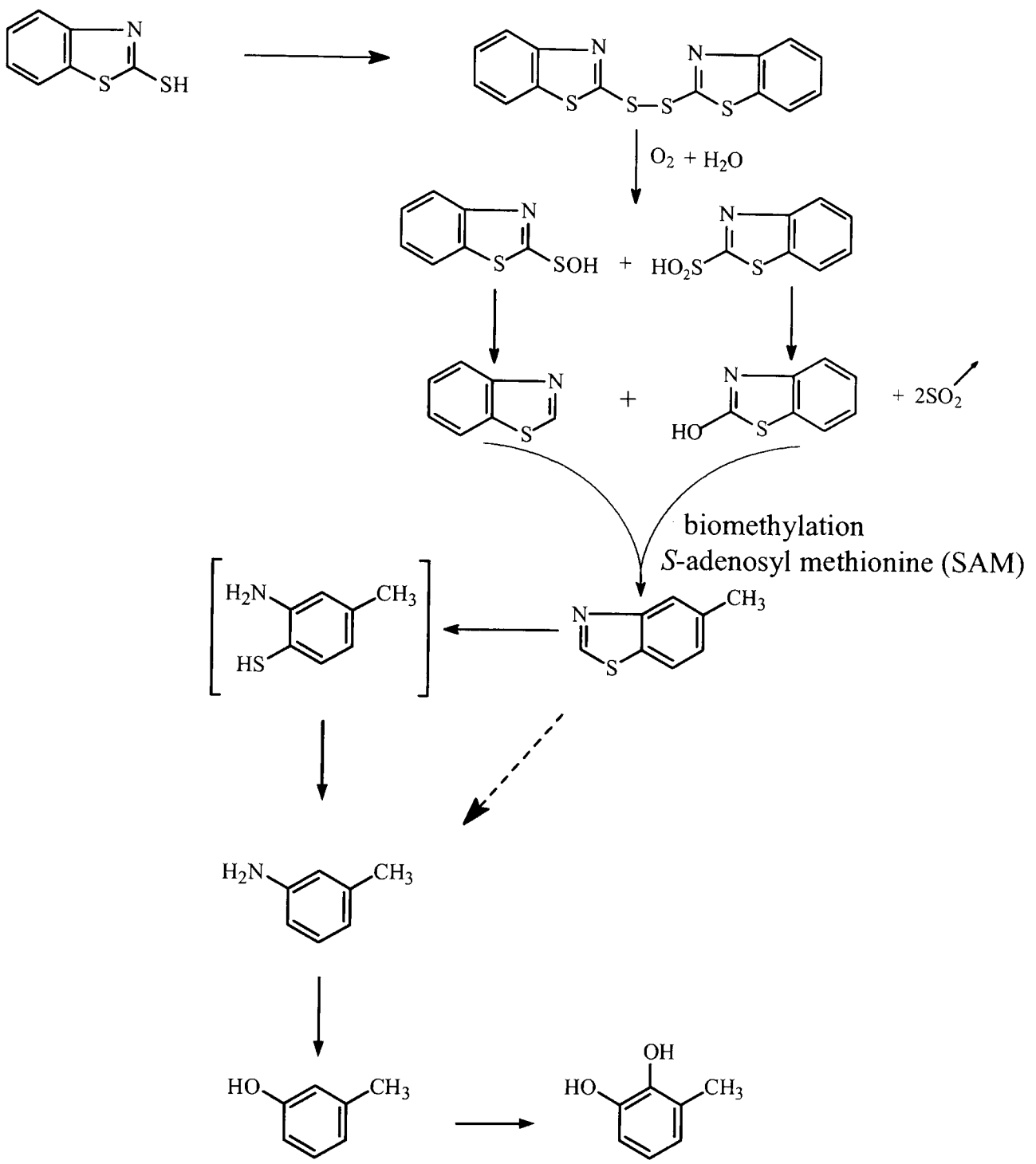


Figure 4.19 Full proposed pathway for MBT biodegradation at 100 mg/l.

Although no BTSA was observed during metabolisation of MBT at 50 mg/l, it was assumed that at the increased concentration this metabolite would be generated. The results of the two experiments suggest that the metabolite BTSA is not along the fate pathway for MBT up to concentrations of 100 mg/l *in this biological system.*

The presence of *m*-toluidine is confirmatory of the methylation scheme leading to 5-MeBTH. The lack of an authentic standard of 5-MeBTH made it difficult to confirm the identity of the component labelled as 5-MeBTH. However, it is strongly supported by the presence of the metabolites *m*-toluidine, *m*-cresol and 3-methylcatechol which are unlikely to be present without 5-MeBTH as a precursor.

4.3.5 Benzothiazole-2-sulphonic acid

Several research groups (Knapp, 1975; Mainprize *et al.,* 1976; De Vos *et al.,* 1993b; De Wever and Verachtert, 1994) have reported that benzothiazole-2-sulphonic acid (BTSA) is not biologically degradable and thought to be recalcitrant though not toxic.

In this study, the BTSA was fed to the biological reactor at 100 mg/l for a period of ten days during which samples were taken and analysed by HPLC.

4.3.5.1 Results for BTSA degradation

Analysis of the first 24 hour effluent sample indicated that BTSA removal to an extent of 99% apparently occurred very quickly as no significant levels of BTSA were detected. This may have been a result of adsorption onto the floc surface, a phenomenon which is well known. However, within 24 hours significant breakdown had taken place with the production of a series of metabolites. The most significant of these were BTH and MBT and an unidentified polar compound eluting very early in the chromatogram. Phenol was also present at high levels, probably due to the mutual inhibition of the biomass by the presence of high levels of both BTH and MBT (Figure 4.20).

By the end of the second day (48 hours), both the BTH and MBT levels continued to increase along with phenol and the unidentified polar compound. 2-Hydroxybenzothiazole appeared at low levels along with a second unidentified polar compound. Identification of this second polar compound was difficult as a consequence of co-elution with the first polar compound (Figure 4.21).

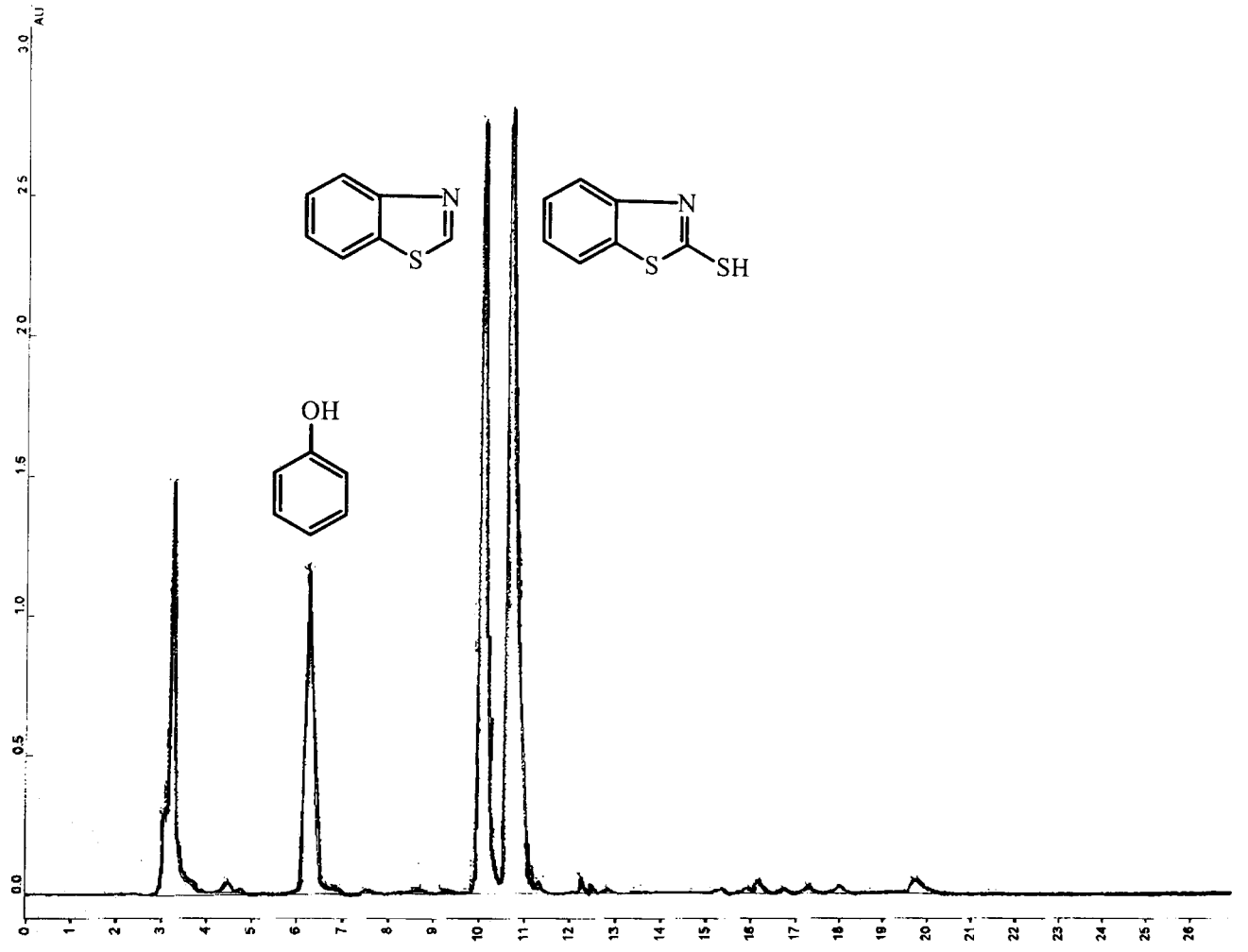


Figure 4.20 BTSA HPLC 1. The first 24 hours.

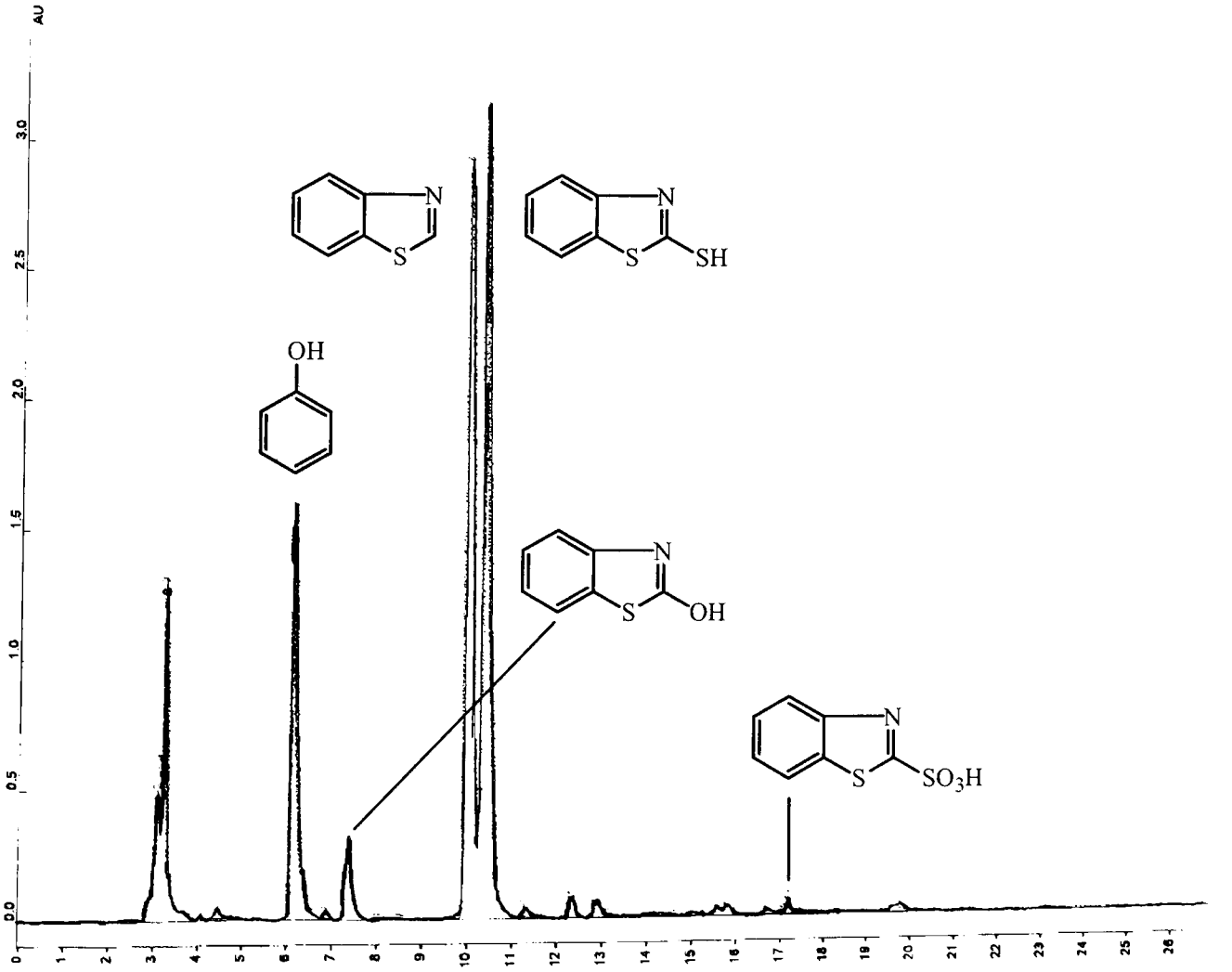


Figure 4.21 BTSA HPLC 2.

After three days all metabolites were still found to have increased but without the detection of any new metabolites. Phenol removal was seen to recover by the end of day four, with levels of BTH and other metabolites still increasing. The second co-eluting unidentified polar compound was no longer detectable.

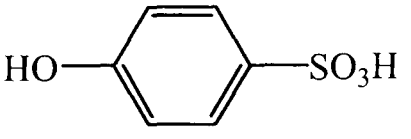
From day five onwards, the level of the unidentified polar compound eluting at the front of the chromatogram appeared to have stabilised while all other metabolites continued to increase with time, phenol removal also showing signs of inhibition.

4.3.5.2 BTSA removal mechanism

From the data obtained in this study, it is difficult to determine the exact pathway for BTSA removal. It would appear to have a very rapid initial step with the exocyclic sulphonic acid bond being broken to simultaneously yield BTH and MBT. This step may also be mediated via the production of the unidentified polar compound eluting at the front of all the chromatograms. The metabolite BTOH only appears some 24 hours after the initial biomass inoculation with BTSA and is seen to develop rapidly thereafter. Since neither BTH nor MBT are seen to reduce in concentration during the experiment while the BTOH is increasing, this would suggest that the source of the BTOH is neither BTH or MBT but may be the BTSA.

The finding of this study is that BTSA is biodegradable, which is in contradiction to the findings of other workers. Furthermore, removal is rapid and complete with the production of four significant metabolites.

A tentative identification of the previously unidentified polar compound eluting at 3.2 mins in all of the chromatograms is based on comparison with a sample of 4-hydroxybenzene-sulphonic acid obtained from the Aldrich-Sigma Chemical Company. The retention time and UV spectra of the standard are very similar to the unknown polar compound.



4-hydroxybenzenesulphonic acid

It is conceivable that this compound is generated by interaction of BTSA with phenol resulting in sulphonation of the phenol in the *para* position. This compound is not observed in the degradation of any other thiazole and is therefore linked specifically to the BTSA breakdown.

On this basis BTSA removal appears to be rapid and may proceed via two mechanisms, one in which the BTH and MBT are both simultaneously generated and the other in which anion exchange occurs between phenol and the BTSA. The anion exchange between BTSA and phenol also gives rise to BTH within the biomass and could explain the high levels of observed BTH. It is difficult to say which process occurs first or is the dominant mechanism.

BTOH is generated approximately 24 hours into the experiment. It is not conclusive that the BTOH is a product of BTSA transformation. It has been shown in BTH metabolisation studies (Section 4.3.1) that BTOH does not appear in the pathway for BTH removal. It is however found along the MBT pathway as determined in the MBT metabolisation studies (Section 4.3.4.1).

4.3.6 2-(4-morpholinothio)benzothiazole

This experiment was carried out using activated sludge previously exposed to a mixture of benzothiazoles not containing morpholine or derivatives of morpholine. 2-(4-Morpholinothio)benzothiazole (MBS) was fed to the biomass of the reactor at two concentrations, 50 and 100 mg/l, sequentially over a ten-day period, a total of twenty days.

Samples were analysed by HPLC (using Method 1) and GCMS for the presence of expected metabolites. The samples for HPLC were simply filtered to remove any insoluble matter (biomass) to prevent fouling of the HPLC system. The GCMS samples were processed via liquid–liquid extraction (LLE) and concentrated to 1 cm^3 using a Zymark closed-cup turbovap concentrator. Analysis via GCMS utilised hexadecane as the internal standard for quantification of detected analytes. HPLC quantification was carried out using an external standard.

4.3.6.1 Results for MBS degradation

Analysis of the effluent collected following the first 24 hours of MBS metabolisation at 50 mg/l clearly showed that no MBT or BTSA was generated, though a few unidentified metabolites were detected. Two components in the HPLC analysis were tentatively identified as benzothiazolyl morpholinyl sulphoxide (BTSOMOR) and sodium benzothiazole-2-sulphinate ($BTSO_2Na$) (Figure 4.22).

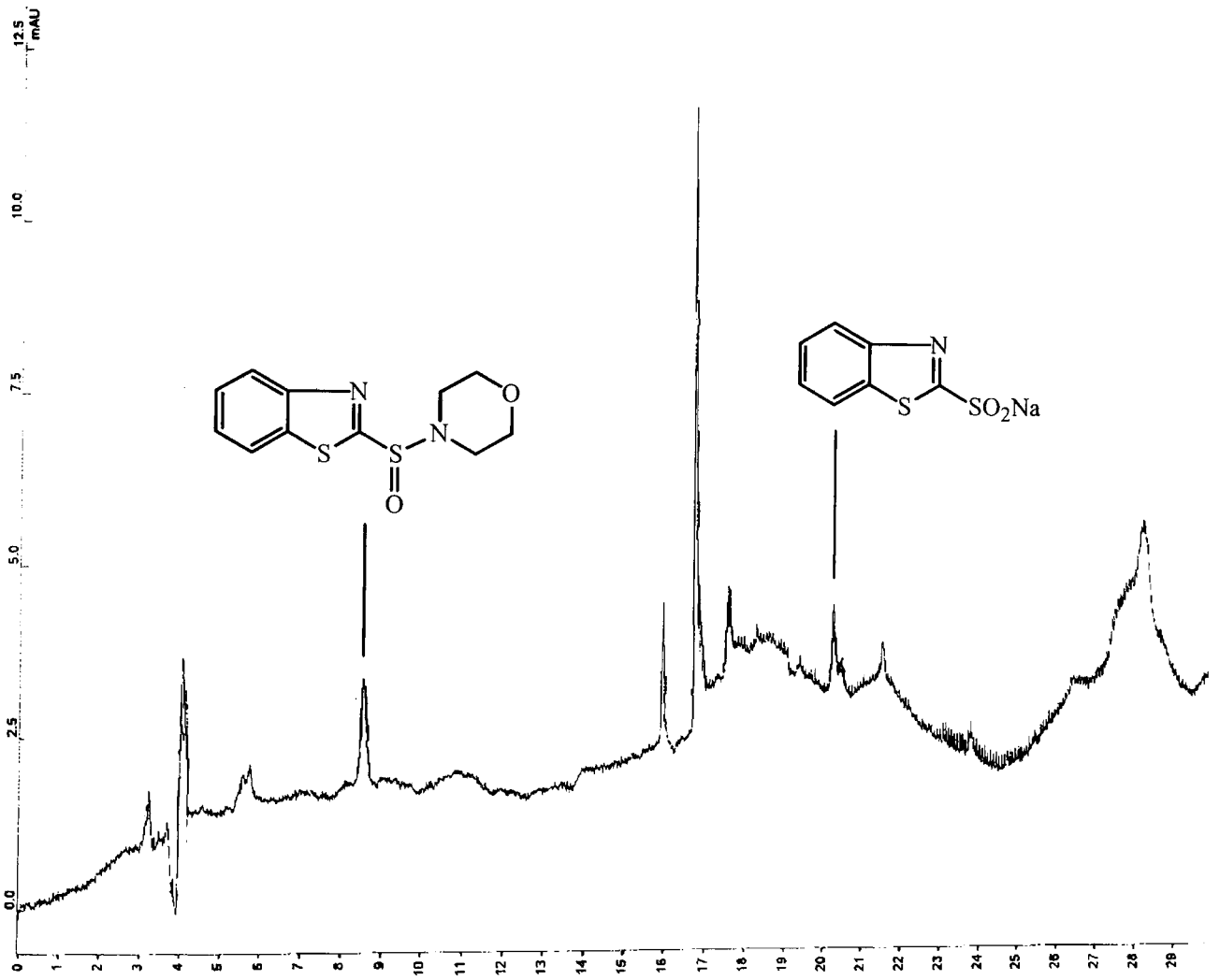


Figure 4.22 MBS HPLC 1. The first 24 hours.

By the end of day two no MBT was detectable in the effluent from the reactor. Levels of other metabolites still continued to increase slowly.

The metabolite MBT was first detected in analysis of effluent collected after three days into the experiment. This would suggest that the initial step in MBS removal involves progressive oxygenation of the exocyclic sulphur atom to give a sulphoxide, a sulphone, and eventually the sulphonic acid (BTSA). The intermediate step in this pathway is the

cleavage of the S–N bond between the benzothiazole and morpholine to yield $BTSO_2Na$ and morpholine.

The slower activation step of S–N cleavage with H_2 reduction to yield MBT and morpholine occurs approximately 48 hours after the initial inoculation of the biomass with MBS (Figure 4.23).

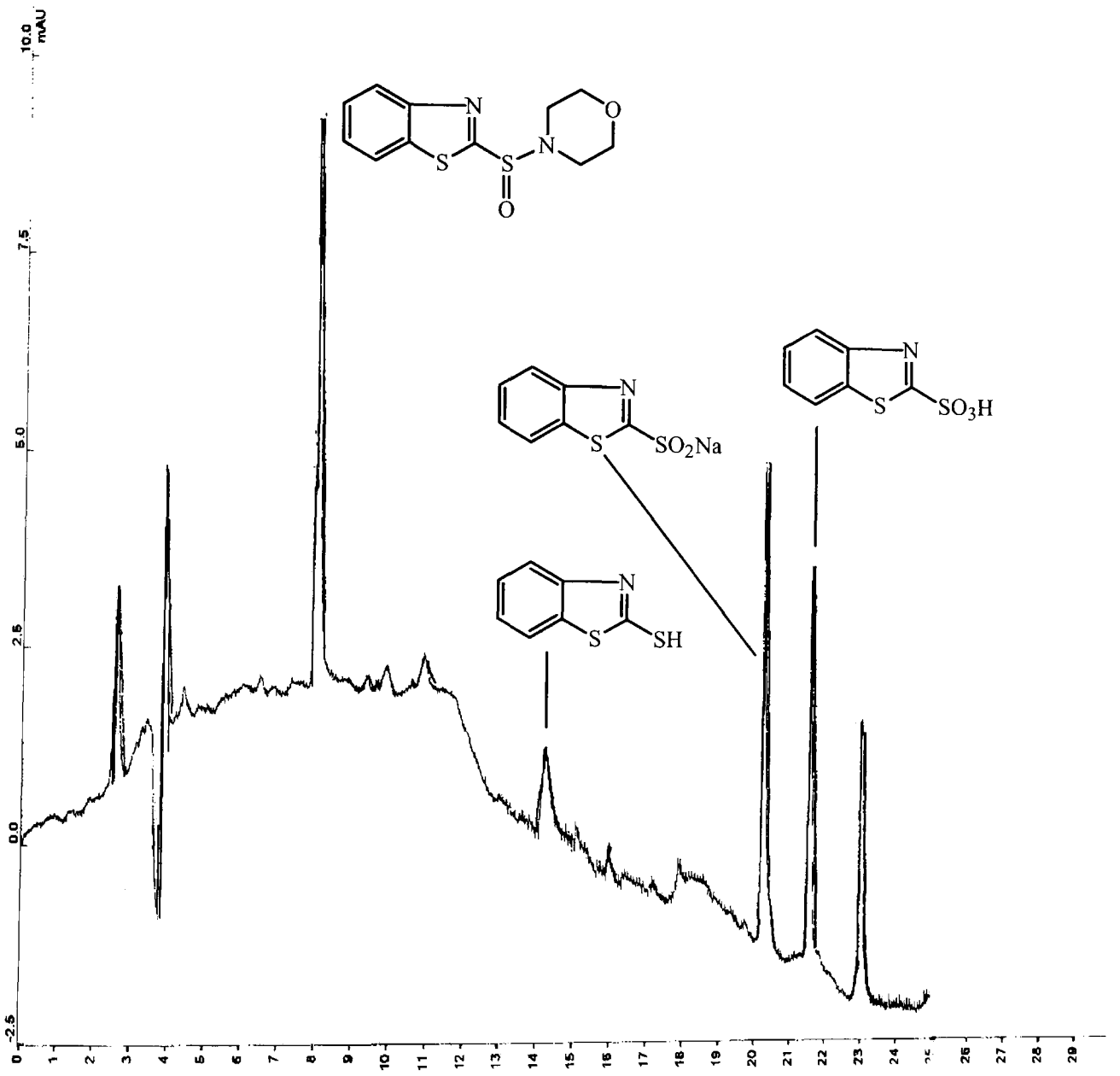


Figure 4.23 MBS HPLC 3. 72 Hours of MBS metabolisation.

GCMS analysis of effluent for the third day of the experiment showed the presence of the two metabolites $BTSOCH_3$ and $BTSO_2CH_3$ at microgram levels.

The metabolites MBT and $BTSO_2Na$ continued to increase over the remaining days of the experiment. The other detected metabolites either decreased slightly in concentration or

stabilised as in the case of the $BTSOCH_3$ and $BTSO_2CH_3$. The MBT concentration was seen to peak at 7.2 mg/l by the end of day eight.

At the end of day ten the MBS concentration was increased in a fresh batch of feed, from 50 mg/l to 100 mg/l. The feed rate was kept constant at 3.5 cm^3/min, the rate for all the previous experiments. This produced an immediate response within the first 24 hours: the metabolites MBT and $BTSO_2Na$ were both very significantly increased in concentration in the effluent from the reactor. BTSOMOR levels were seen to fall slightly whereas the $BTSO_3H$, $BTSOCH_3$ and $BTSO_2CH_3$ levels were observed to plateau.

Previous GCMS analysis had not detected the presence of MBT. However, following the increase in MBS concentration in the feed to the biomass, levels of MBT in the effluent were such that GCMS detection was possible. MBT does not chromatograph well on a DB5MS column at low levels, and consequently under the conditions used MBT only became detectable at levels above 20 mg/l. In Figure 4.24, the chromatogram shows clearly the reduction in the level of BTSOMOR in the effluent from the reactor.

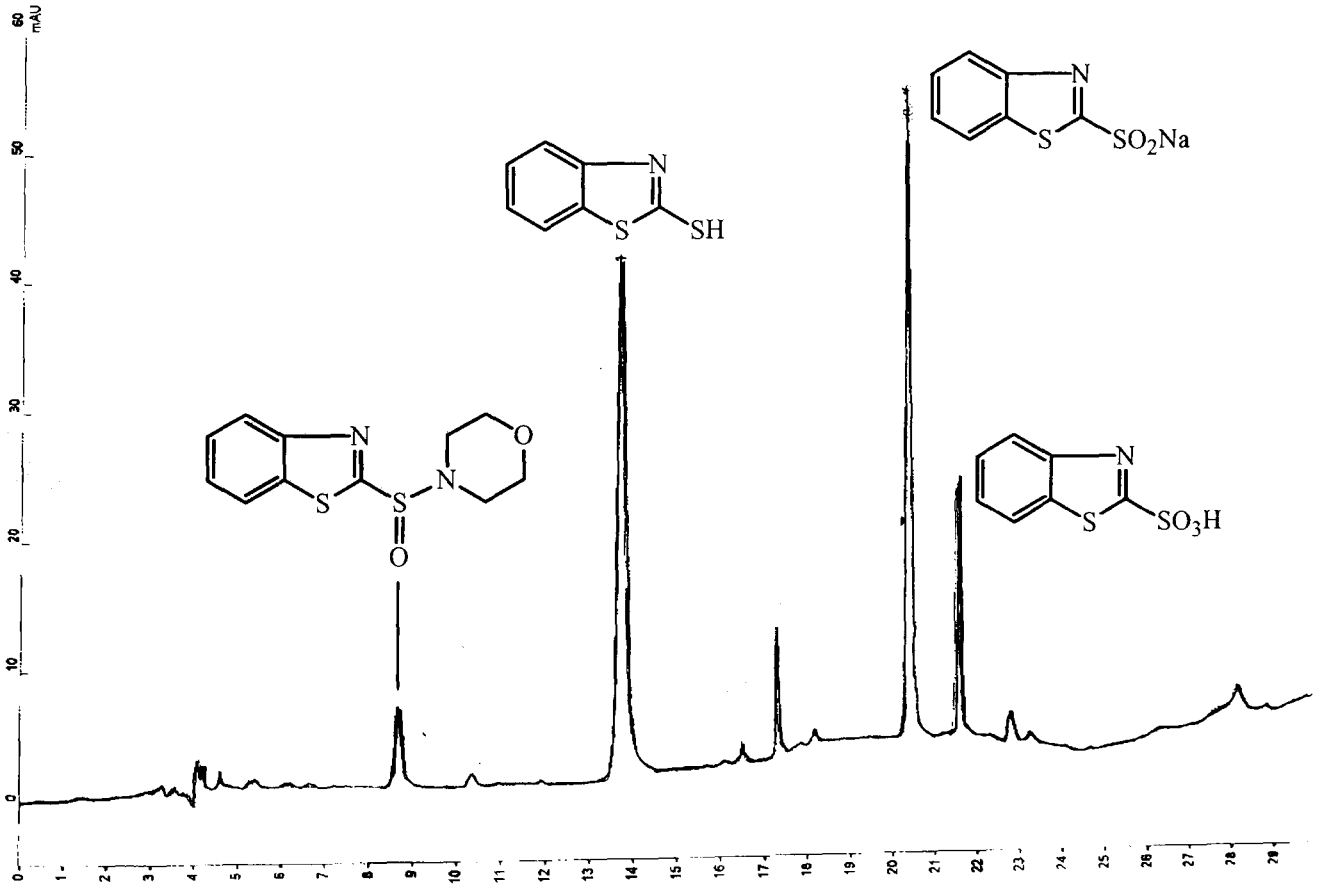


Figure 4.24 MBS HPLC 11. The first 24 hours at 100 mg/l MBS.

4.3.6.2 Fate pathway for MBS

It was thought hitherto that the biodegradation of MBS in an activated sludge reactor proceeded via progressive oxidation of the exocyclic sulphur atom to give a sulphoxide, a sulphone and eventually the sulphonic acid (BTSA), the BTSA being formed following cleavage of the S–N bond between the benzothiazole and morpholine (Figure 4.25).

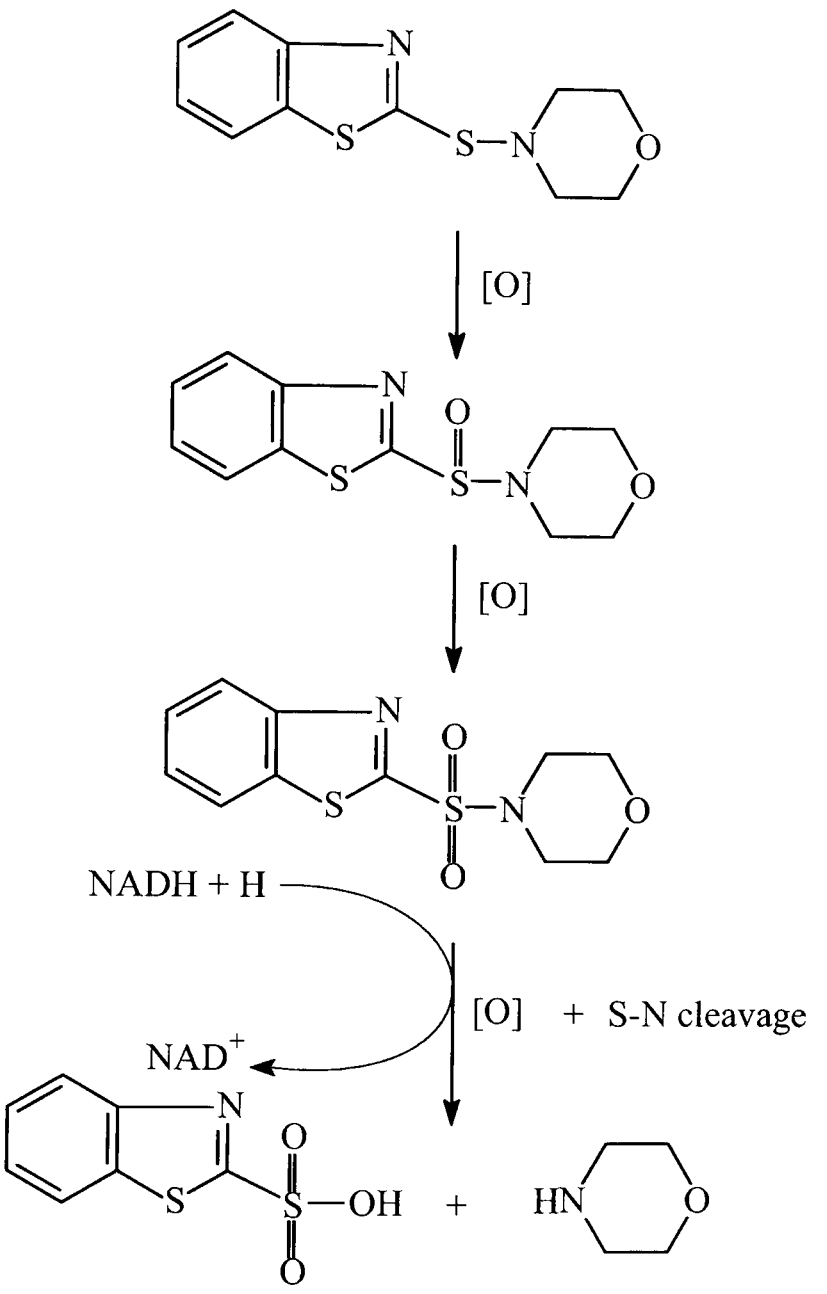


Figure 4.25 Progressive oxidation to yield BTSA.

However, it now appears from the present study that this pathway only proceeds as far as the first step with the formation of BTSOMOR instead of undergoing further oxidation. It would also appear that biomethylation precedes any further oxidation steps, with the release of morpholine (Drotar and Fall, 1985). The methylated product then undergoes a series of oxidations resulting in the production of benzothiazole-2-sulphonic acid (BTSA).

The morpholine liberated during the biomethylation step and S–N cleavage is lost via two routes:(a) via the well-documented glycolate pathway (Knapp *et al.*, 1982; Swain *et al.*, 1991; Poupin *et al.*, 1998) and (b) through formation of the dead-end metabolite dimorpholino ketone (DMK).

No $-SO_2MOR$ derivatives were detected thus confirming that the OS–N bond is broken prior to further oxidation to the $-SO_2MOR$. On the basis of both the GCMS and HPLC analyses, the scheme shown in Figure 4.26 is proposed as a mechanism for the removal of MBS by a mixed culture activated sludge system.

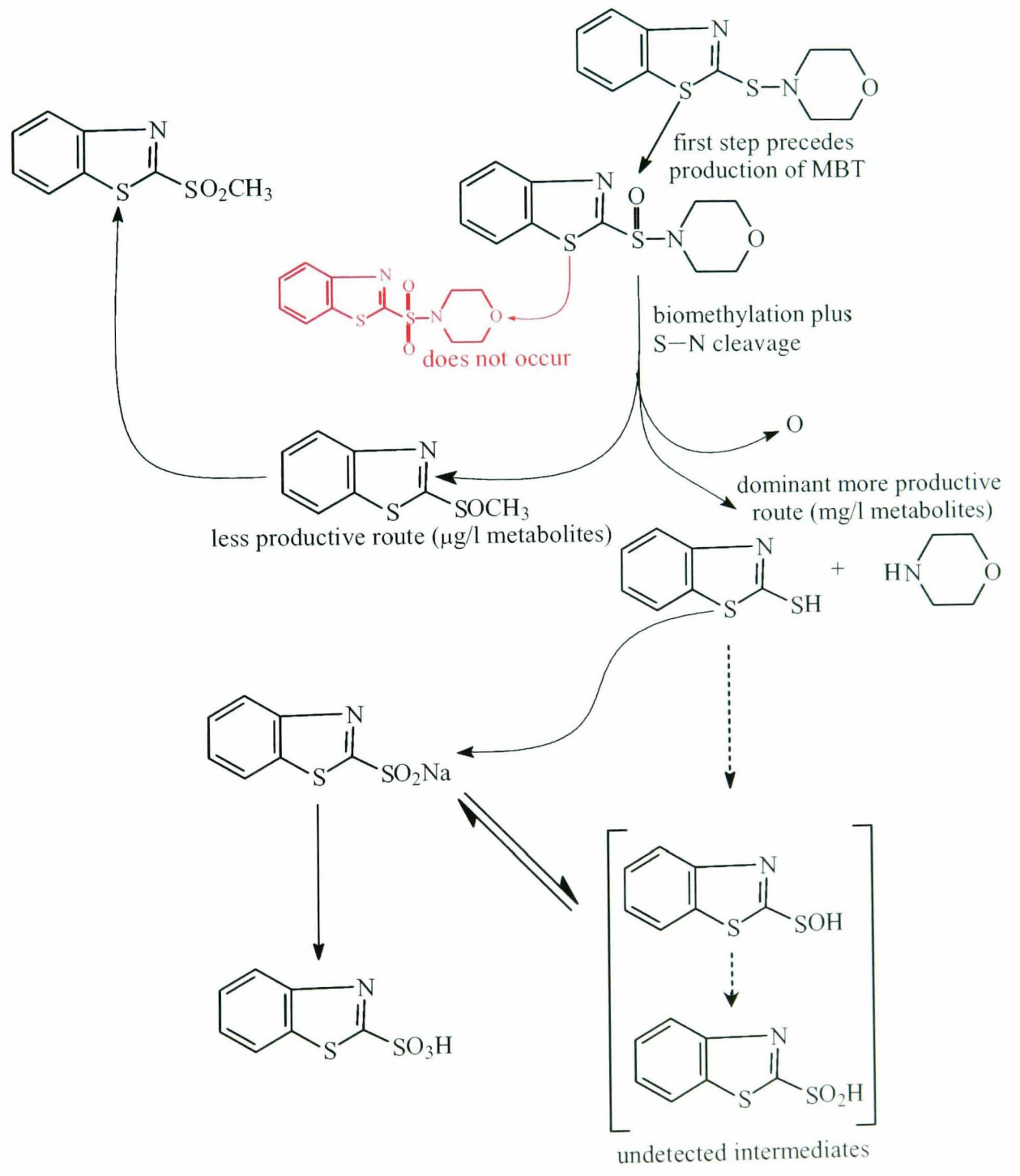


Figure 4.26 Proposed mechanism for MBS removal by a mixed culture activated sludge.

Note that morpholine is not detected in the analysis because it is too polar to be isolated by dichloromethane extraction as in LLE; direct injection HPLC analysis fails also because morpholine has a UV absorption which lies under that of the acetonitrile used in the eluent for the analysis.

4.4 Binary substrate biodegradation studies

All the work described so far has been based on the study of single substrate (thiazole) interactions with the biomass. This section details the results obtained when the biomass is inoculated with two substrates simultaneously. The experimental details were the same as that for the single substrate studies.

4.4.1 2-Mercaptobenzothiazole/benzothiazole-2-sulphonic acid

It has been demonstrated that 100 mg/l MBT had no significant toxic effects on the performance of the biomass; the same has also been shown for BTSA at 100 mg/l (see Sections 4.3.4.2 and 4.3.5).

For the following studies the synthetic feed mixture was spiked with 25 mg/l MBT and 100 mg/l BTSA and fed as normal to the biomass of reactor R3 over a ten-day period. All samples were collected in discrete 24 hour aliquots; samples were prefiltered prior to analysis to remove any traces of solids.

4.4.1.1 Results of MBT/BTSA metabolisation

Analysis of effluent collected after 24 hours of the experiment clearly demonstrated that MBT/BTSA at this level was exerting biological inhibition, since BTSA residues were present at higher levels in the effluent compared to those observed in BTSA-only degradation studies. Traces of BTH and BTOH were also observed in the sample indicating that biodegradation was proceeding and not totally inhibited (Figure 4.27).

By the end of the second day both MBT and BTOH levels had increased confirming that some mutual inhibition of the biomass was being exhibited by the MBT/BTSA mixture. BTSA and BTH concentrations were similar to those found in the first 24-hour sample.

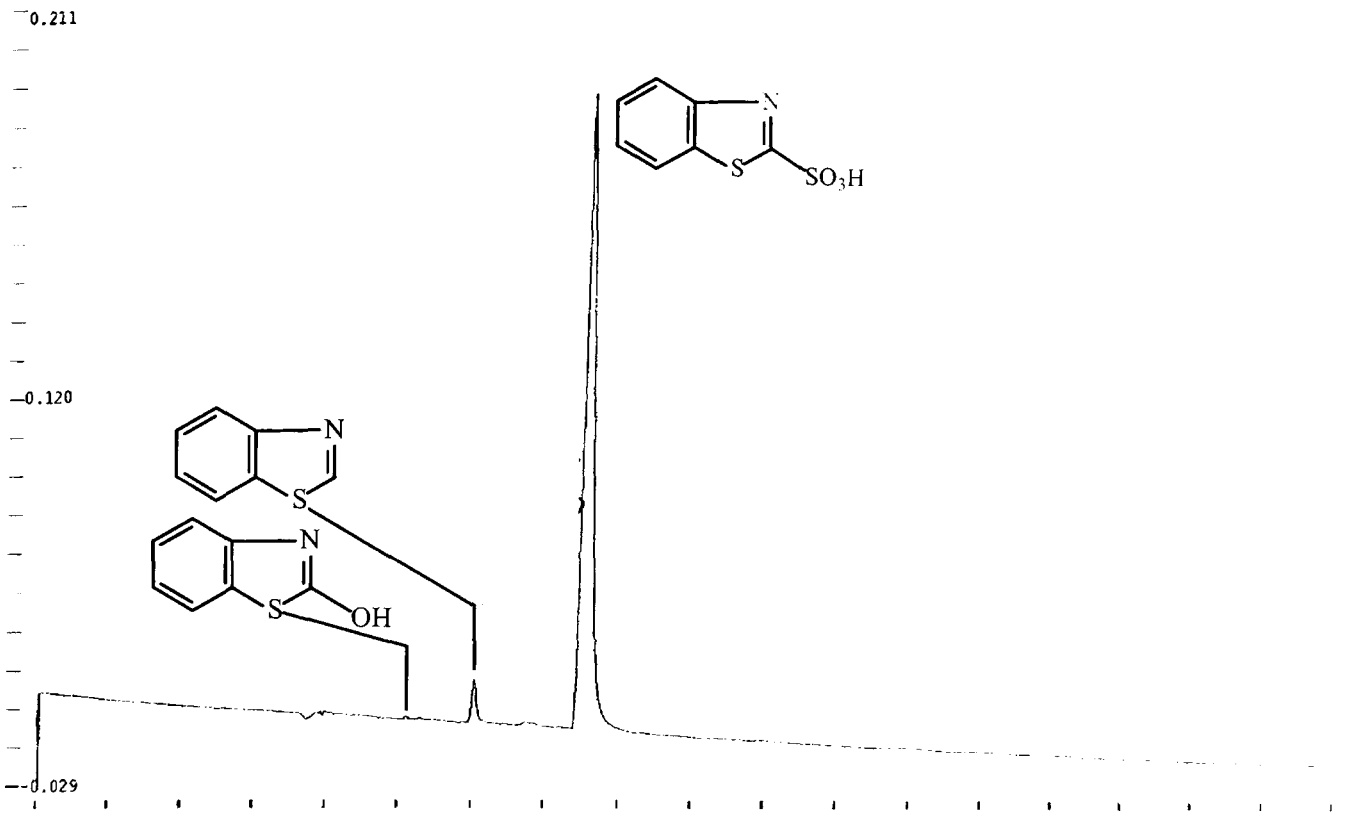


Figure 4.27 MBT/BTSA HPLC 1. The first 24 hours.

Analysis of effluent collected at the end of the third day was similar to that for day two, in that the levels of BTOH and MBT were seen to increase slightly while BTH and BTSA appeared to have stabilised.

Analysis of effluent collected after four days showed that the onset of BTOH removal had begun while the rate of MBT increase somewhat slowed. BTSA degradation was observed to be increasing. BTH was no longer detectable in the effluent sample after four days.

Analysis of effluent collected after five days confirmed that biological activity was increasing and that the initial inhibitory effect exerted by the MBT/BTSA was wearing off. BTSA levels continued to fall with a corresponding increase in the level of MeBTH observed in the effluent. MBT levels continued to increase, suggesting that the specific bacterium responsible for MBT removal was being inhibited by the metabolites being generated. The presence of MeBTH was further proof that the biomass was recovering from the effects initially exhibited by both MBT and BTSA.

Analysis of effluent collected at the end of day nine of the experiment showed a slight trace of $BTSO_2H$ and BTOH. The level of MeBTH was almost half of the BTSA concentration, with MBT levels also slightly increased. By the end of the ten-day experiment the MeBTH concentration was over half of the BTSA level. Furthermore, the

residual MBT:BTSA ratio was almost 1:1. Table 4.2 summarizes the chromatographic data for each species determined during the experiment with associated charts in Figure 4.28.

Table 4.2 MBT/BTSA metabolisation chromatographic data (peak area response).

day	1	2	3	4	5	6	7	8	9	10
MBT	8958	36225	51067	52147	73248	96142	107523	140391	143714	163916
BTSA	224803	239592	271926	273781	221623	152844	140219	153184	59490	162671
MeBTH	0.5	0.5	0.5	0.5	0.5	0.5	73801	92118	84769	94805
$BTSO_2H$	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	7741	2504
BTOH	100	11360	17548	14997	13261	9053	4119	1711	1670	1249
BTH	2376	1819	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

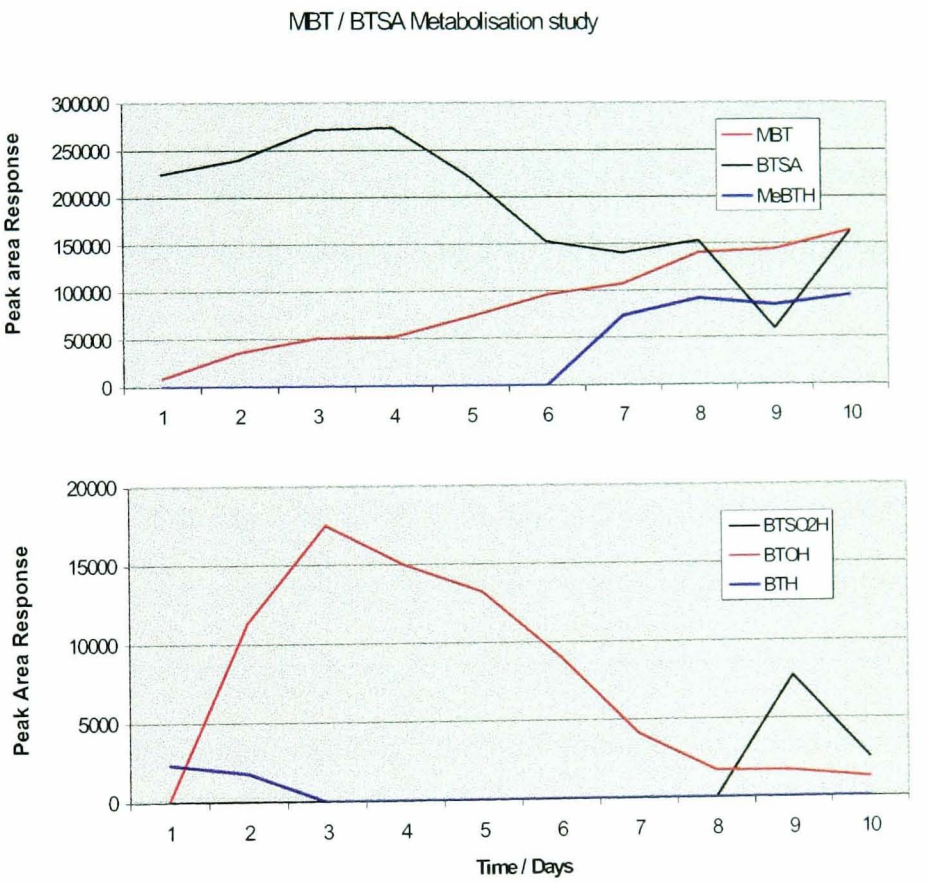


Figure 4.28 MBT /BTSA metabolisation charts.

4.4.1.2 Conclusions from MBT/BTSA metabolisation study

In previous experiments where BTSA was fed to the biomass (see Section 4.3.5), it was shown that BTSA removal was complete and very fast. On the other hand MBT has been shown to be only partially biodegradable at concentrations up to 50 mg/l (Section 4.3.4).

The results of this study of the binary mixture indicate that MBT at levels below 50 mg/l (25 mg/l) with 100 mg/l BTSA have a deleterious synergism when fed to the biomass used in this study, demonstrated by the lack of BTSA biological degradation.

It has also been demonstrated that during biodegradation of MBT alone at low levels, both MeMBT and BTOH featured in the metabolic pathway. In the binary mixture BTOH does not feature very strongly as a product of MBT breakdown and MeMBT was not generated at all. This would suggest that a different mechanism was in operation for the MBT degradation in the presence of BTSA, one in which the formation of MeBTH is favoured over the methylation of the MBT.

Similarly with BTSA breakdown, BTH, MBT and BTOH were observed to be metabolic products. Very little BTH/BTOH was generated during the course of this experiment, whereas the metabolite MeBTH was produced (which was not the case with breakdown of BTSA alone). This can be explained if it is assumed that BTH generation was slow in the presence of MBT with methylation to yield the MeBTH analogue being relatively fast, resulting in little free BTH being detected in the effluent while being fed the mixture of MBT/BTSA.

The lack of any further biodegradation to yield any catechol or 3-methylcatechol further confirms that the system has been severely affected with a resulting reduction in biological activity, though running the experiment for a longer time would have been necessary to be certain. Quantitative data for analysis of the effluent collected during this experiment also supports the claim that biological activity had been reduced. The results presented in Table 4.3 show how the bulk of the organic material (MBT/BTSA fraction) was still present after ten days of continuous operation.

Table 4.3 MBT/BTSA metabolites. (+/– 0.10 mg/l)

Time/days	Phenol mg/l	BTH mg/l	MBT mg/l	2-MeBTH mg/l	BTSA mg/l	%residual MBT/BTSA
0	<0.10	<0.10	<0.10	None detected	<0.10	Not applicable
1	<0.10	0.1	2.2	None detected	79	65.04
2	21.3	0.12	8.8	None detected	81.4	72.22
3	24.6	<0.10	10.6	None detected	82.5	74.5
4	32.9	None detected	12.4	None detected	96.6	87.2
5	28.1	None detected	12.7	None detected	96.7	87.52
6	2.5	None detected	12.3	None detected	71.4	66.96
7	0.95	None detected	13.7	19.7	63.3	77.36
8	0.15	None detected	19.2	24.5	53.6	77.84
9	0.28	None detected	34.1	25.3	39.5	79.12
10	0.1	None detected	39.8	27.6	31.2	78.88

The residual MBT/BTSA fraction remained fairly constant throughout the whole of the experimental period, demonstrating that the biomass had been inhibited by the introduction of the MBT/BTSA mixture.

4.4.2 2-Hydroxybenzothiazole/benzothiazole-2-sulphonic acid

For this experiment, the base synthetic feed matrix was spiked with 25 mg/l BTOH and 100 mg/l BTSA. The HPLC analytical conditions had to be modified to take account of a change in LC pump module. The original Varian Vista 5500 LC pump failed and was replaced with a new motor and pump head. Unfortunately this gave rise to separation problems when using the gradient profile for the MBT/BTSA analysis, so a modified method was devised to rectify the situation (see Chapter 2, HPLC Method 7).

4.4.2.1 Results from BTOH/BTSA metabolisation study

The effluent collected following the first 24 hours of the experiment was found to contain traces of an unidentified metabolite eluting at t_R 3.3 mins, along with a number of less significant unidentified metabolites. However, no BTSA was detectable at that stage (Figure 4.29).

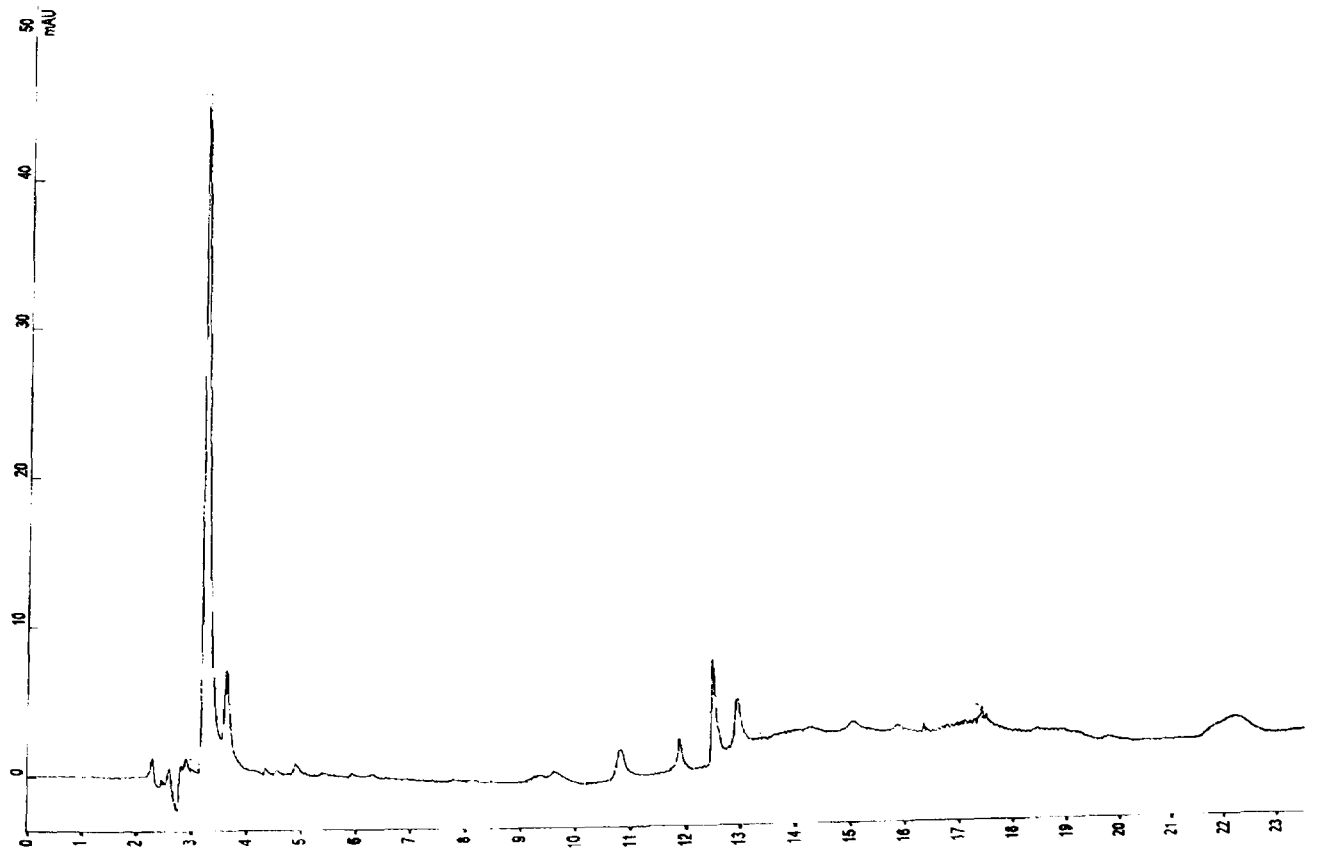


Figure 4.29 BTOH/BTSA HPLC 1. The first 24 hours.

Traces of BTSA were found to be present in the effluent collected after 24 hours. The unknown polar intermediate eluting at t_R 3.3 mins was still present, although at a much lower level than that detected in the first 2 hour sample. BTOH was still notably absent in the effluent and appears to be completely broken down by the biomass.

Analysis of effluent collected after four days of the experiment showed that the polar intermediate at t_R 3.3 mins was being steadily metabolised and/or not being generated by the biomass. At the same time BTSA levels continued to increase at a steady rate. Other metabolites continued to be generated albeit at low levels. Over the course of the remaining six days, the levels of BTSA continued to increase. A new unidentified polar intermediate eluting at t_R 24.8 mins was detected on day five of the experiment and varied in concentration but was present to the end of the experiment. The concentration of the polar compound eluting at t_R 3.3 mins continued to decrease over the remainder of the experiment. Table 4.4 gives the peak area response data for the two major components detected during the experiment with the associated chart in Figure 4.30.

Table 4.4 BTOH/BTSA Peak area response data.

Days	1	2	3	4	5	6	7	8	9	10
Polar compd t_R 3.0 mins	50615	26503	24700	26177	25577	23143	22057	18731	16011	13647
BTSA	0.5	6446	35453	76598	79221	85263	87641	91174	95632	102146

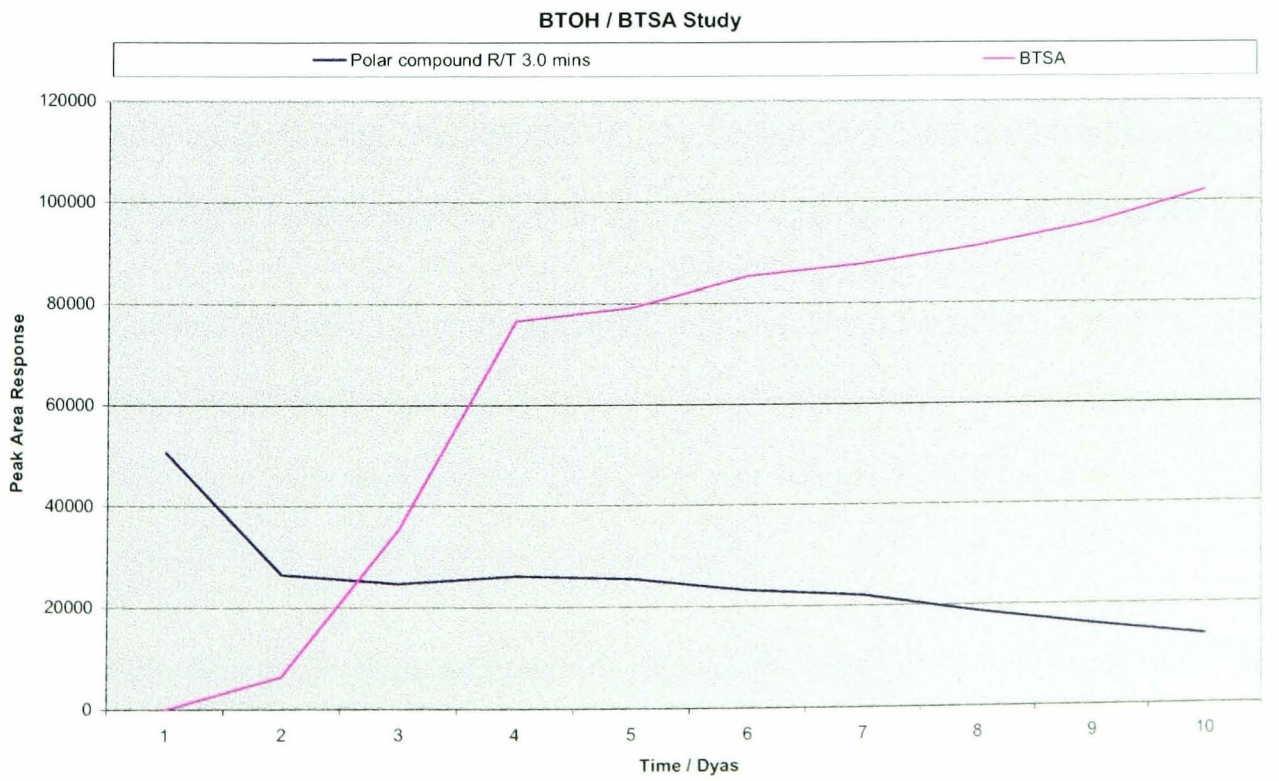


Figure 4.30 BTOH/BTSA metabolisation chart.

4.4.2.2 Conclusions from BTOH/BTSA study

It would appear that while BTSA removal was inhibited in the presence of BTOH, the same was not true for BTOH in the presence of BTSA. BTOH removal was rapid and complete, as shown by the lack of any BTOH in any of the samples collected during the course of this experiment.

BTSA removal was initially unaffected but after 24 hours was rapidly inhibited. This may be the result of toxic effects due to the presence of the polar intermediate at t_R 3.3 mins or simply surface adsorption onto the biological floc.

4.4.3 Benzothiazole/2-hydroxybenzothiazole

The synthetic feed was spiked with 25 mg/l each of BTH and BTOH and fed onto the biomass of biological reactor over a ten-day period. All analytical conditions applied were the same as for the BTOH/BTSA experiment.

4.4.3.1 Results of BTH/BTOH metabolisation study

In the experiments using BTH and BTOH as sole substrates for the biomass, it was observed that both were only partially degraded with the production of a series of metabolites. Both substrates undergo an initial ring scission step, followed in the case of BTH (but not BTOH) by methylation (Sections 4.3.1.1 and 4.3.2.1).

Analysis of the sample collected following the first 24 hours showed both BTH and BTOH to be rapidly removed from the system. A series of polar intermediates were produced with t_R 2.8, 3.3 and 3.7 mins, together with a minor metabolite at t_R 10.7 mins (Figure 4.31).

The analysis of effluent collected after a further 24 hours indicated that BTH was being inhibited to a small degree as traces of this substrate were observed along with MBT and a number of very minor metabolites. The polar intermediates eluting at t_R 2.8, 3.3 and 3.7 mins are still present and at slightly elevated levels.

Analysis of effluent samples collected over the following few days showed little change in the composition or concentration of the various metabolites present. However, the BTH concentration was observed suddenly to increase after four days, whereas the

concentration of MBT was almost constant showing only a slight upward trend. It is clear from the chart that the system had recovered after eight days of inoculation with the binary mixture BTH/BTOH.

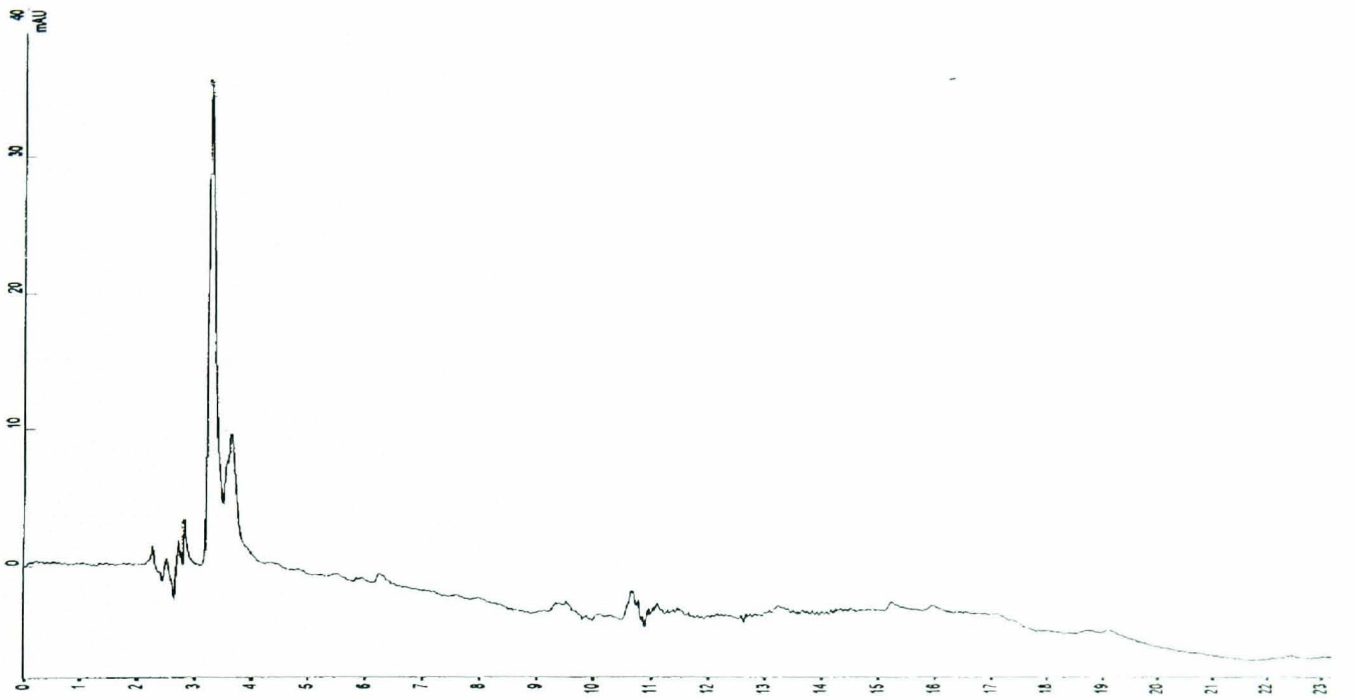


Figure 4.31 BTH/BTOH HPLC 1. The first 24 hours.

Table 4.5 gives details of all analytical data for the samples analysed and Figure 4.32 is the corresponding chart.

Table 4.5 Peak area response for BTH/BTOH study.

Days	1	2	3	4	5	6	7	8	9	10
Polar compound t_R 3.0 mins	46200	55658	56263	54641	55119	57063	59176	60767	62651	19736
BTH	0.5	2832	2963	3116	32561	33411	35261	43834	11868	9034
MBT	0.5	4946	5163	5273	5448	5718	6076	6623	7193	6051

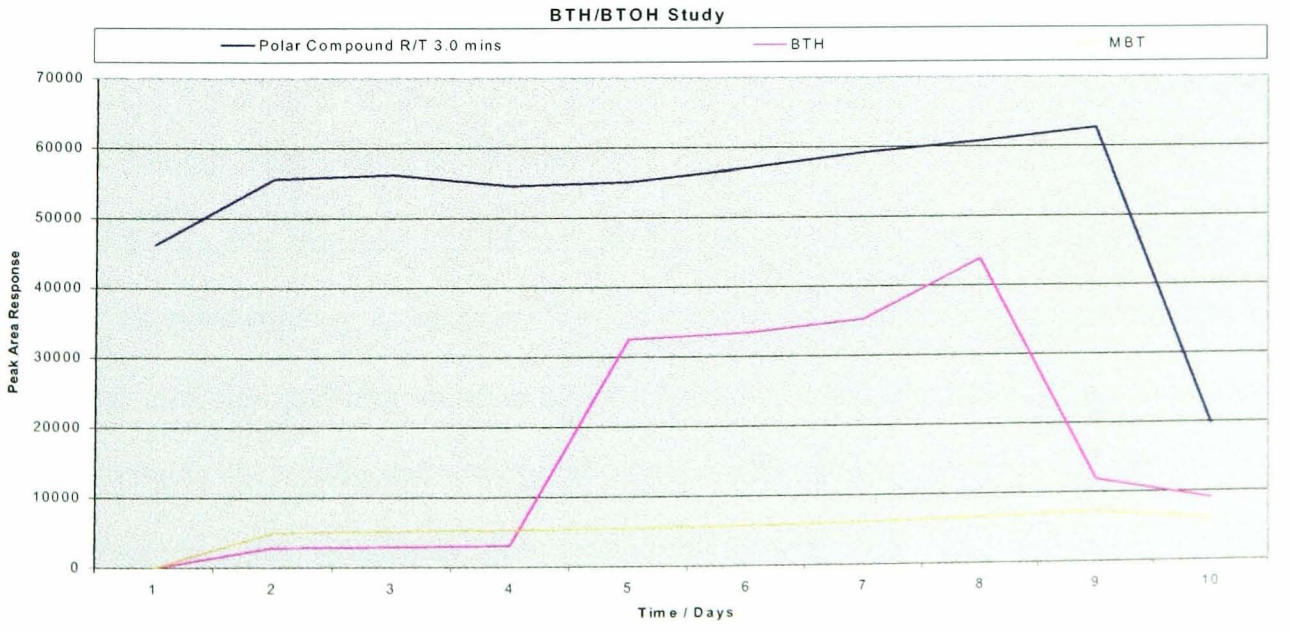


Figure 4.30 BTH/BTOH metabolisation chart.

4.4.3.2 Conclusions from BTH/BTOH metabolisation study

It would appear that initially the biomass degraded the incoming thiazoles quite readily leaving little residual BTH or BTOH. This may also be due to adsorption on to the floc surface, which persists for only a relatively short time, *ca* 24 hours. However, at some time between 24 to 48 hours a toxic threshold was reached, at which point the biological removal of BTH was inhibited, as indicated by the presence of traces of BTH in the effluent sample analysed at the end of the second day of the experiment.

The presence of MBT is difficult to explain, since it does not feature along the metabolic pathways for either BTH or BTOH. It does show that binary substrate systems stimulate the biomass in such a way that modified metabolic pathways come into play where a specific metabolite *e.g.* MBT is produced for substrates that do not normally generate such a metabolite.

It was demonstrated by the findings of this experiment that BTOH removal was not inhibited in the presence of BTH. In contrast, BTH removal was shown to proceed at a faster rate in the presence of BTOH than found in the single substrate studies with BTH when removal was very slow (see Section 4.3.1).

4.4.4 2-Mercaptobenzothiazole/2-hydroxybenzothiazole

Both substrates (MBT and BTOH) were spiked at 25 mg/l into the synthetic feed and fed on to the biomass over a ten-day period. Samples were collected and analysed in a similar manner to the previous experiments.

4.4.4.1 Results from MBT/BTOH study

Figure 4.33 shows that both MBT and BTOH were present at significant levels after 24 hours of metabolisation. A series of unidentified metabolites were present confirming that biological activity had not been unduly inhibited, although BTOH metabolisation does appear to have been affected by the presence of the MBT. Analysis of effluent collected after the second day confirmed that biological activity was still very active in that both substrates continued to be degraded with the co-production of metabolites BTH and other unidentified polar intermediates.

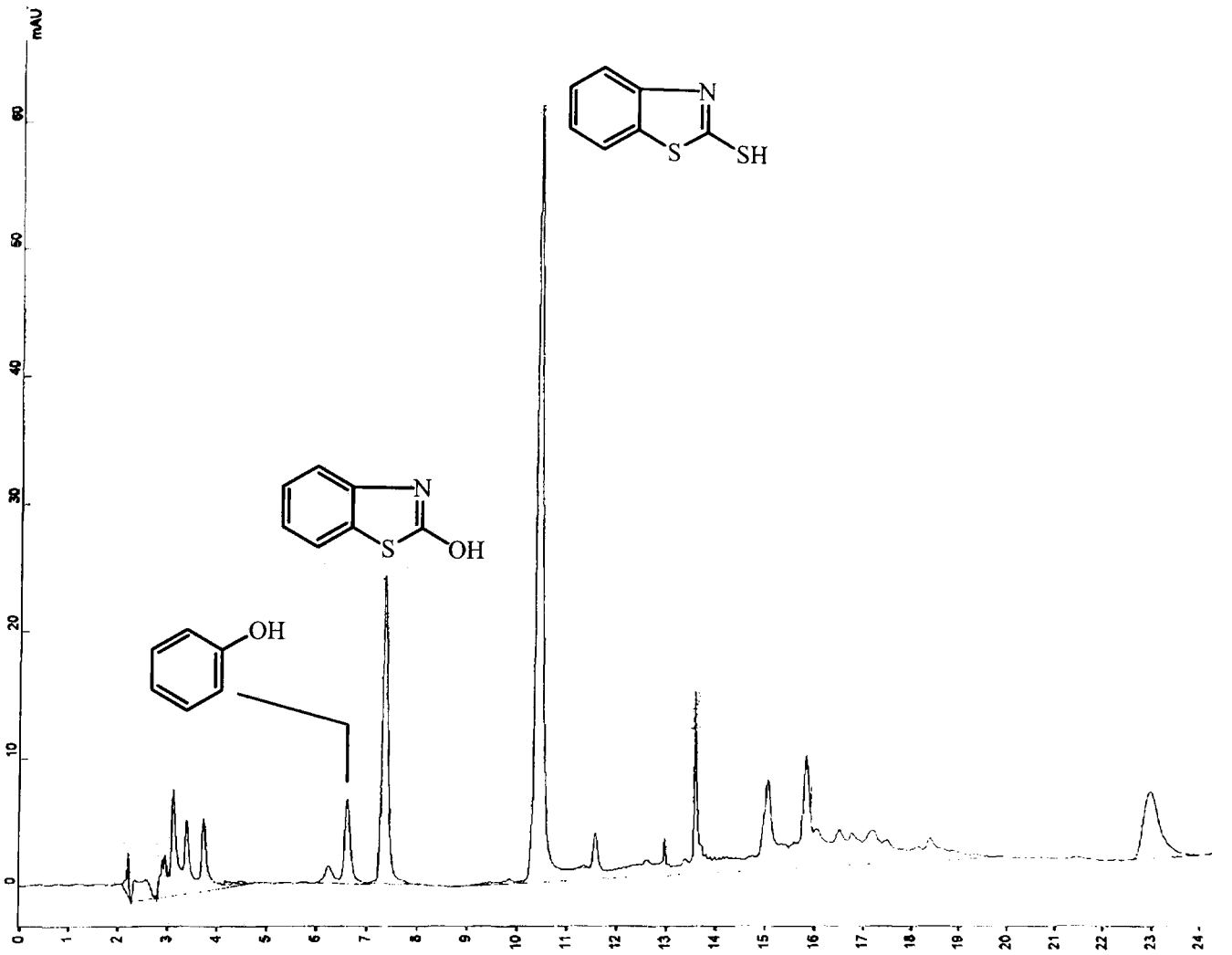
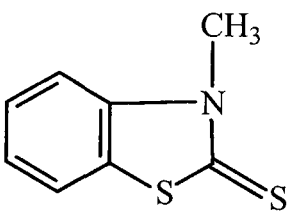


Figure 4.33 MBT/BTOH HPLC 1. The first 24 hours.

Effluent samples collected at the end of days 3 and 4 showed nothing significant in the way of generated metabolites; however, by the end of day five, 3-methylbenzothiazole-2-thione (MeBTT) was detected.



3-methylbenzothiazole-2-thione (MeBTT)

The presence of MeBTT can be explained if one considers the possible presence of an alternative methyl transferase not involving *S*-adenosyl methionine (SAM) (Drotar *et al.*, 1985). This general class of transferases has been shown to be more tolerant of toxic load as indicated by the onset of production of MeBTT at day five of the experiment. In contrast the SAM mediated methylation pathway was inhibited for a further 5 days with MeMBT production not occurring until the final day of the experiment.

Other metabolites still continued to be generated but only at low levels in comparison to the principal products, namely phenol, BTH, MBT, and MeBTT; BTH was still present though at low concentrations compared with these.

There were no major changes in the composition of the samples collected for the remaining few days of the experiment. The analysis of effluent from the last day of the experiment however confirmed the presence of new metabolites along with increased levels of BTH and the two polar intermediates at t_R 3.3 and 3.7 mins. The metabolites eluting at t_R 12.0 and 16.6 mins had also increased significantly towards the end of the experiment. Table 4.6 summarizes the analytical data collected during the course of the MBT/BTOH metabolisation study and the associated chart is shown in Figure 4.34.

Table 4.6 Peak area response for MBT/BTOH study.

Days	1	2	3	4	5	6	7	8	9	10
Phenol	7332	10513	12663	16872	21946	22367	20131	17614	15205	12788
BTOH	26865	50605	51123	50874	50667	48667	41215	38656	29248	11140
BTH	0.5	2056	2063	2135	2216	2305	2416	2647	8254	11183
MBT	76429	89441	91021	92796	94998	90632	85113	77032	59653	32675
N-MeMBT	0.5	0.5	0.5	0.5	9614	9973	10239	10876	11463	12039
2-MeMBT	1279	3572	5262	6893	8052	9061	10116	10864	11386	12094

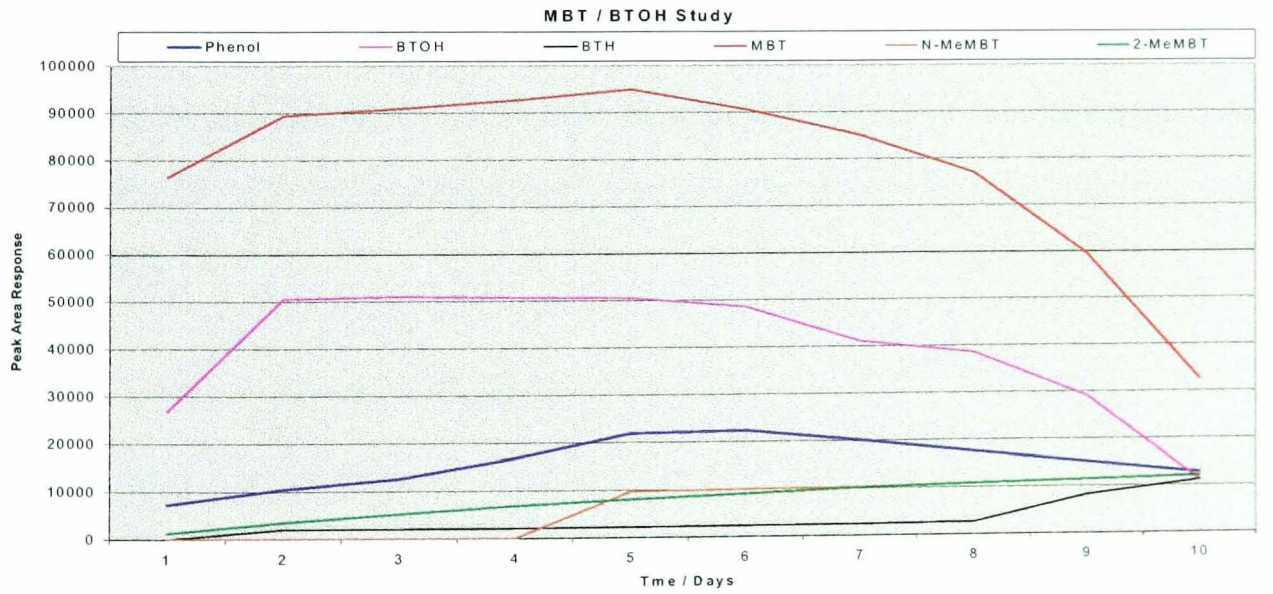


Figure 4.34 MBT/BTOH metabolisation study.

4.4.4.2 Conclusions from MBT/BTOH study

The concentration of BTOH and MBT residues in the effluent steadily increased over the course of the experiment, which would suggest that they were mutually inhibiting the biomass resulting in their slow removal from the system. However, analysis of the effluent collected at the end of the experiment indicated that the system was recovering since the concentration of both BTOH and MBT was lower than that observed at the early stages of the experiment. Careful examination of the chart for the MBT/BTOH metabolisation revealed that MBT recovery began around day five of the experiment and that the rate of MBT removal increased towards the end of the experiment.

All metabolites were significantly higher by the end of the experiment, which would confirm that the biomass was recovering from the effects of mutual inhibition exerted by the MBT/BTOH substrate system.

It has been shown in previous investigations (Mainprize *et al.,* 1976; Liu *et al.*, 1983; De Wever and Verachtert, 1994) that MBT was only partially degraded in activated sludge systems. Chudoba *et al.* (1977) investigated benzothiazole degradation by adapted activated sludge and found that BTOH was degradable. The results of this study are in agreement with these findings in that MBT is degradable but less so than BTOH. In work by De Wever and Verachtert (1994) it was shown that MBT was the main component responsible for toxic inhibition of biological systems; this is also confirmed by the findings of this study.

4.4.5 2-Mercaptobenzothiazole/benzothiazole

The synthetic feed matrix was spiked as in previous experiments with 25 mg/l of the substrates under investigation and fed on to the biomass of reactor R3 over a ten day period. Effluent samples were collected as before and analysed for the presence of residual MBT/BTH and expected metabolites by HPLC.

4.4.5.1 Results of MBT/BTH study

Single component studies of both MBT (Section 4.3.4) and BTH (Section 4.3.1) have shown that they are only partially degraded in an activated sludge system.

Analysis of the effluent collected after the first 24 hours of inoculation of the biomass with a feed containing both MBT and BTH confirmed the findings of the earlier single component studies in that significant levels of both substrates were found in the effluent (Figure 4.35).

One other significant metabolite was also observed eluting at t_R 3.5 mins (this metabolite appears in most of the thiazole systems studied so far and has not as yet been successfully identified).

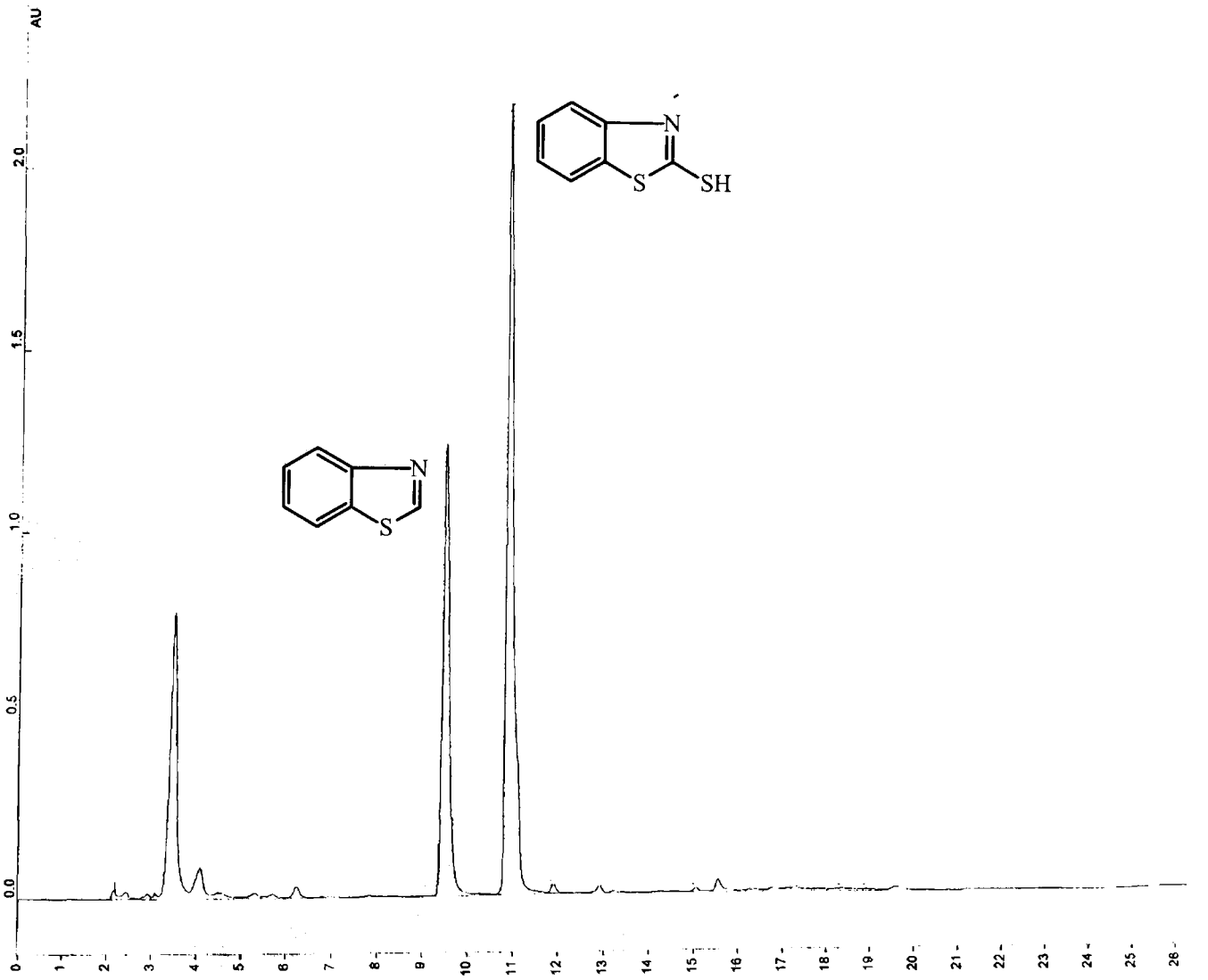


Figure 4.35 MBT BTH HPLC 1. The first 24 hours.

The composition of effluent collected at the end of the second day was shown by HPLC analysis to have changed slightly in that phenol was present at significant levels, confirming that carbonaceous removal was inhibited. The previously unidentified polar intermediate at t_R 3.5 mins continued to increase slightly. Further metabolites were detected including MeBTH, MeMBT and $BTSO_2H$. The peak at t_R 7.8 mins has a spectrum similar to that of BTSOH, although the t_R is slightly shifted. (Figure 4.36).

By the end of the day three, levels of the various metabolites present continued to increase, with BTOH now being detectable. Biological activity was shown to increase at some time between 3 and 4 days, with a marked reduction of all detected species in the effluent, the most significant loss being of MBT. Levels of BTH were observed to fall indicating that metabolism of this substrate was also taking place at a greater rate than initially observed. At the same time, BTOH levels were seen to be increasing.

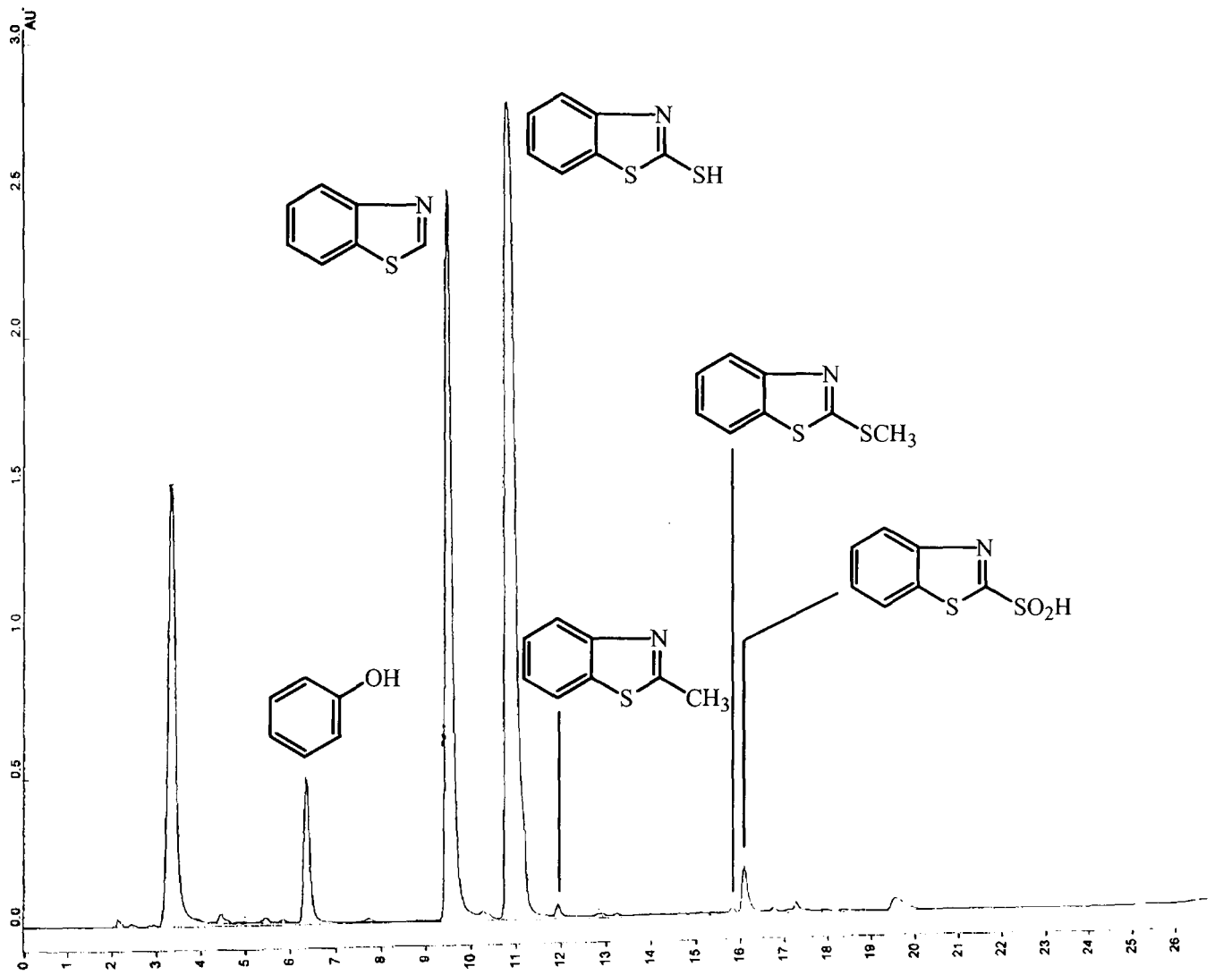


Figure 4.36 MBT BTH HPLC 2.

Analysis of samples collected from day five to day eight showed a steady trend in the change of BTOH with BTH concentration. By the end of day nine BTH was no longer present in the effluent and BTOH concentration continued to increase albeit very slowly. Analysis of the effluent for the last day of the experiment confirmed complete removal of BTH and that BTOH production had slowed down accordingly. Had the experiment run for a longer period we may well have observed the removal of BTOH as in previous experiments. Table 4.7 shows the peak area response analysis data for detected species and the associated charts are shown in Figures 4.37 and 4.38.

Table 4.7 Peak area response data for MBT/BTH study.

Days	1	2	3	4	5	6	7	8	9	10
Polar cmpd t_R 3.0 mins	1117003	2564232	2114861	5221	4188	2748	0.5	7991	6780	8193
BTH	1660466	3542354	3501704	66667	76309	78835	66499	37179	0.5	0.5
MBT	2949094	6138701	6614049	78179	88176	127179	136707	133341	131867	130560
Phenol	0.5	671065	1683501	23940	26661	26351	28692	32579	9911	23482
$BTS0_2H$	0.5	218063	120821	0.5	0.5	0.5	0.5	0.5	0.5	0.5
BTOH	0.5	0.5	13521	6876	11292	19640	32430	53453	53892	54275
MeBTH	0.5	36745	36876	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2-MeMBT	0.5	12658	27158	6012	4538	11169	5923	8421	7475	6211

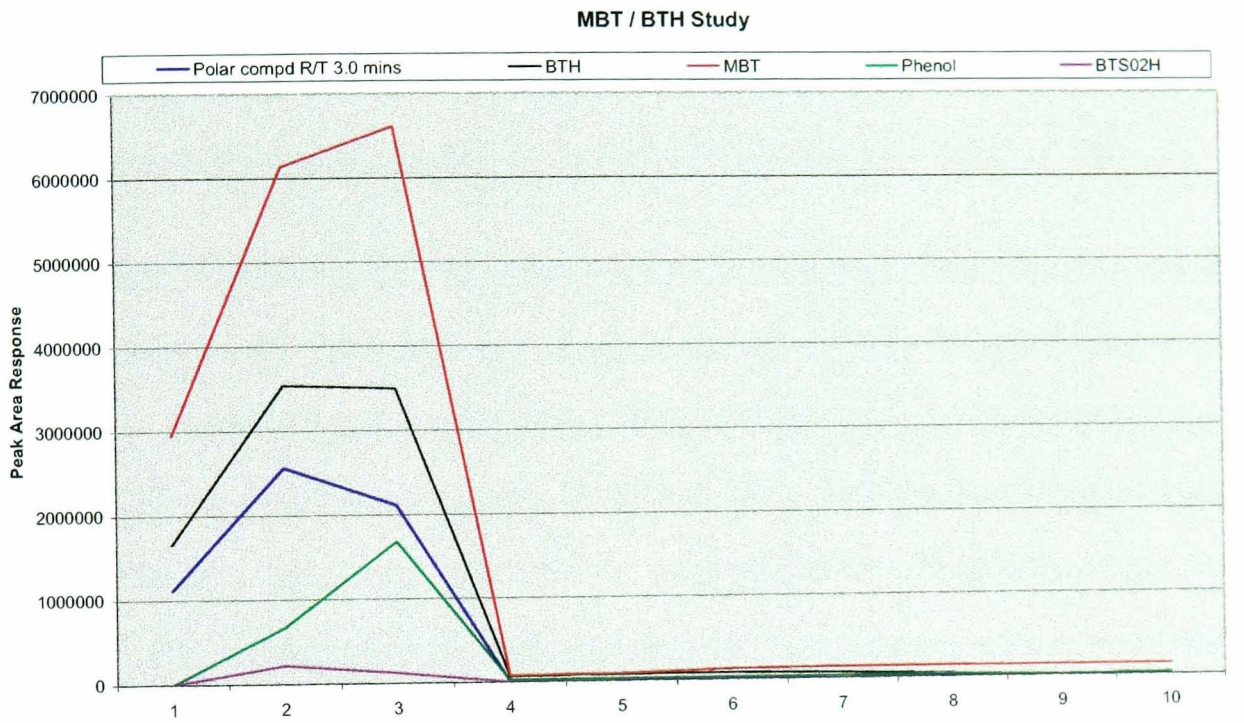


Figure 4.37 MBT/BTH metabolisation study: data for BTH, MBT, phenol and $BTSO_2H$.

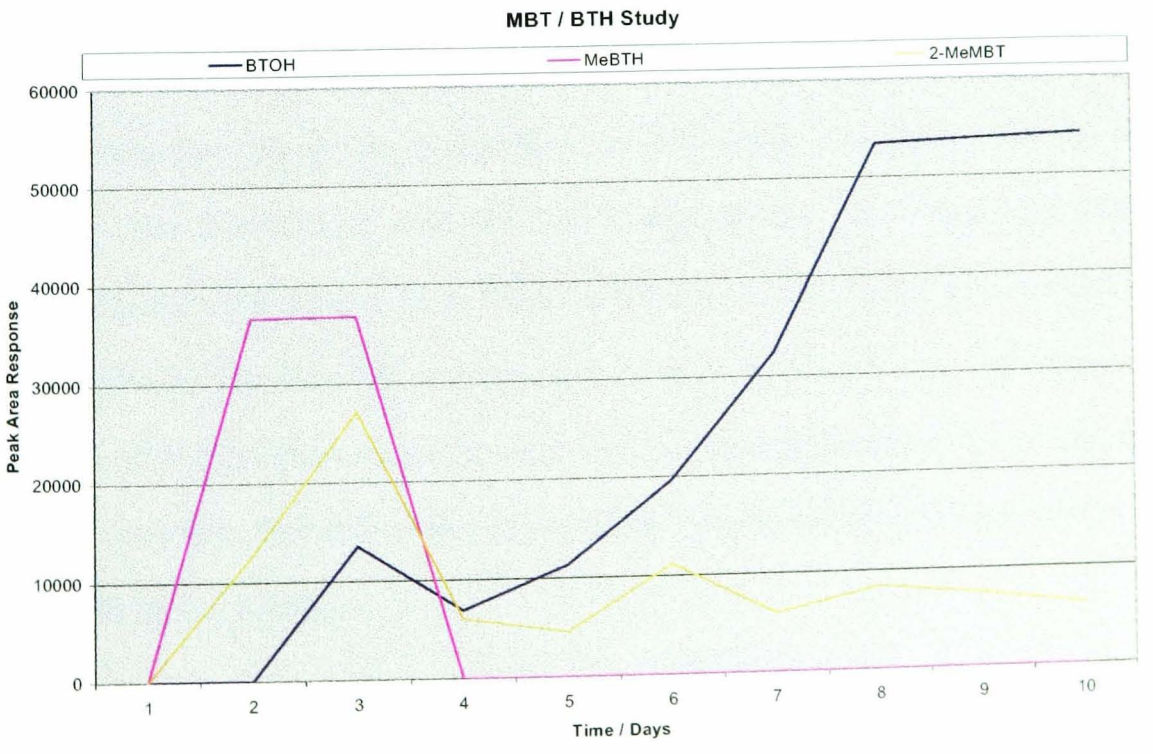


Figure 4.38 MBT/BTH metabolisation study: data for BTOH, MeBTH, and MeMBT.

4.4.5.2 Conclusions from MBT/BTH study

The biomass was initially affected by the introduction of the MBT/BTH feed mixture, in that significant levels of each substrate were present in the effluent from the system. In single component studies of MBT biodegradation one of the major metabolites observed was the species BTOH. The onset of BTOH generation by the biomass in the presence of MBT was 4 days at 50 mg/l and 5 days at 100 mg/l MBT in the feed.

At 50 mg/l MBT, only low levels of BTOH were generated, while at 100 mg/l MBT the generated BTOH is at a level greater than the residual MBT. This establishes that the biological activity was not unduly inhibited by the presence of MBT at 100 mg/l.

In the above experiment the presence of the BTH apparently stimulated the breakdown of MBT to produce BTOH at an earlier stage in the process: BTOH generation was observed after 3 days compared to previous studies where BTOH was not detected until 4 and 5 days respectively. In contrast the levels of BTOH were non-detectable when a mixture of BTH/BTOH was fed to the biomass and BTH removal was initially rapid but rapidly inhibited. This would imply that the growth in BTOH concentration was not due to BTH inhibition. It was also noted that as the level of BTOH increased so the breakdown rate of BTH increased, with no BTH being detectable after 8 days. In studies of BTH alone the removal of BTH was slow and incomplete even after 10 days.

However, it would appear that, as the system became acclimated, the MBT pathway was slowly stimulated with the production of low levels of BTOH. As the MBT to BTOH pathway was established and the MBT degradation rate increased, more BTOH was generated and at the same time MBT inhibition of BTH removal declined with time and the levels of BTH then fell as the substrate was degraded. The final stage of this was the removal of BTOH from the system shown by the reduction in the levels of BTOH in the effluent, towards the end of the experiment, demonstrating acclimation to the high levels of BTOH by the biomass.

4.5 Ternary substrate biodegradation studies

In order to investigate the effects of multiple substrate systems on the biomass performance, two ternary substrate mixtures were studied, namely MBT/BTH/BTSA and BTH/BTOH/MBT. In these studies, the substrates were spiked at 50 mg/l into the synthetic feed matrix used for all previous studies.

4.5.1 MBT/BTH/BTSA

Each of the above compounds was spiked at 50 mg/l into the synthetic feed and fed onto the biomass of reactor R3 for ten days. As in all previous experiments, effluent was collected in discrete 24 hour lots. HPLC analytical conditions for this experiment were the same as those used for the analysis of MBT/BTSA studies in Section 4.4.1.

4.5.1.1 Results of MBT/BTH/BTSA study

The first 24 hour sample was found to contain MBT residues at high concentration along with a lower concentration of BTSA. The BTH was present at even lower levels and phenol was present only at trace concentrations. MeBTH formation had occurred within the first 24 hours and at a relatively high concentration (Figure 4.39).

At the end of the second day no further metabolites were identified, MeBTH and MBT showed signs of being metabolised while BTSA levels continued to increase along with BTH. Carbonaceous removal appears to be unaffected, indicated by the lack of any phenol residues in the effluent, unlike in previous experiments where phenol was present.

Analysis of samples collected for the remaining days of the experiment confirmed the production of BTOH by the biomass. MBT degradation showed little change over the remaining days of the study. Both BTH and MeBTH levels continued to increase while BTSA was degraded by the biomass at a steady rate. Phenol was conspicuously absent throughout the whole of the experiment, confirming that the presence of MBT/BTH/BTSA had no effect on the removal rate of phenol, in fact it would appear to stimulate its removal (see concluding section for a complete discussion of this phenomenon).

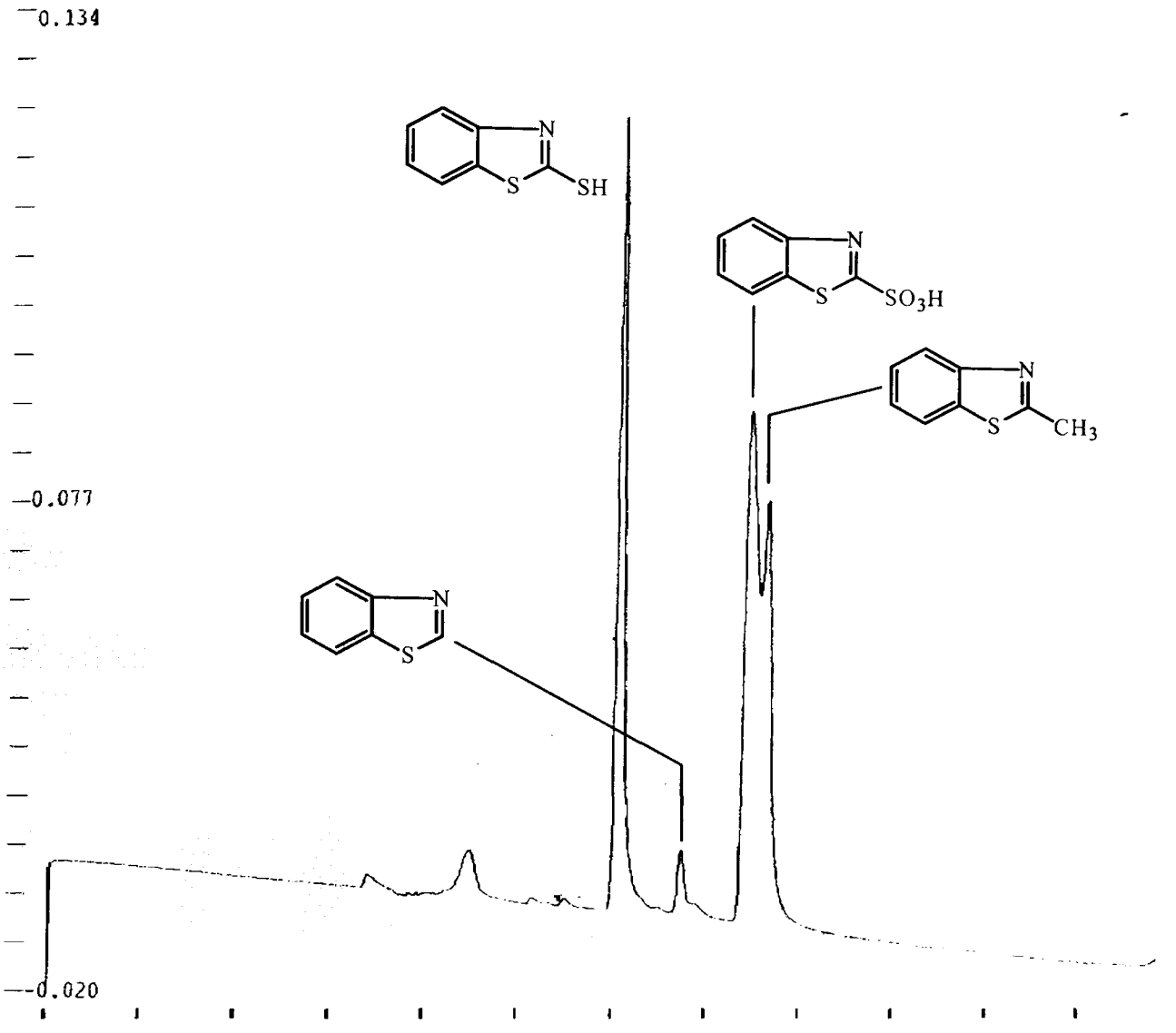


Figure 4.39 MBT/BTH/BTSA HPLC 1. The first 24 hours.

The results of all HPLC analyses are given in Table 4.8 and presented in chart form in Figure 4.40. The chart clearly shows how the various substrates present in the feed are slowly metabolised to produce some very minor unidentified metabolites along with BTOH. Biological activity is clearly adversely affected but not sufficiently so that carbonaceous removal is inhibited, confirmed by the lack of phenol. The presence of BTOH is a good indicator of biological activity in this experiment since it has been shown previously that BTOH is a product of MBT degradation by the specific biomass used in the study.

Table 4.8 HPLC analysis data.

Days	1	2	3	4	5	6	7	8	9	10
BTSA	102154	110747	134424	121096	157615	161105	156235	150865	146098	129350
MBT	91230	85902	73517	88802	79983	77126	72389	78106	83372	89435
MeBTH	70227	91447	71547	109812	106937	111368	109068	113785	122817	147512
BTH	9357	26745	28586	39660	51922	66778	78967	87255	93768	97272
BTOH	0.5	0.5	0.5	544	1025	4117	6077	13657	18663	23659

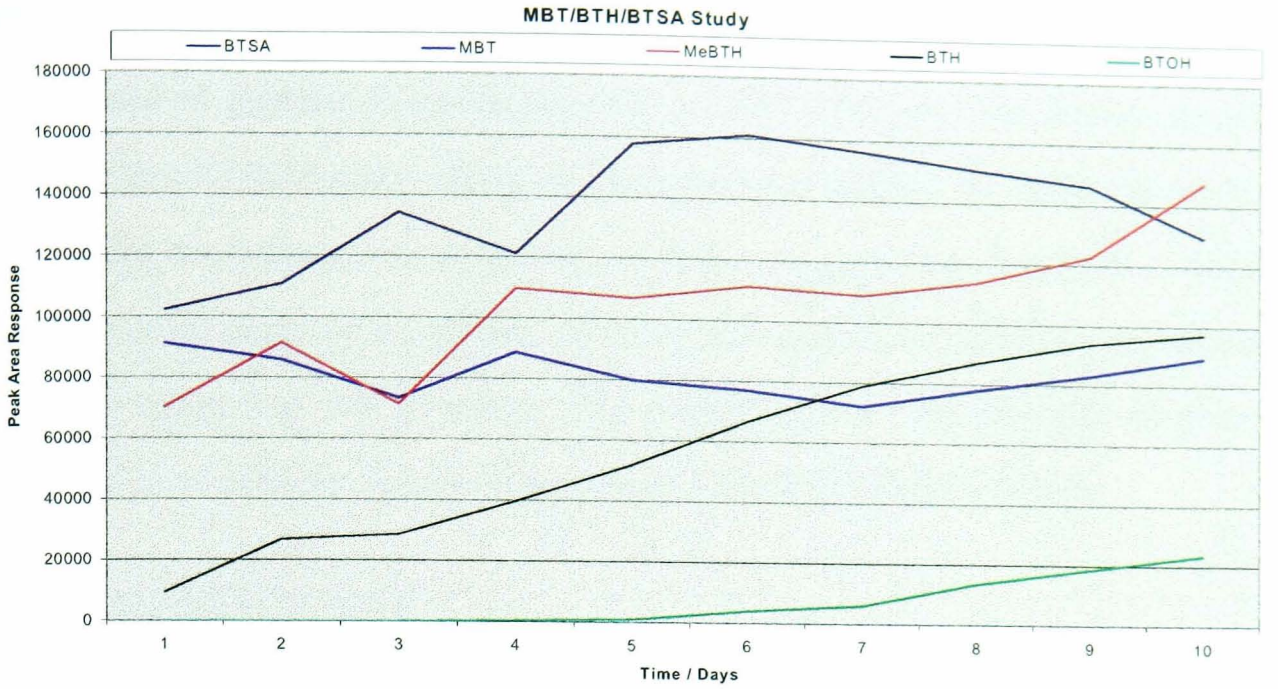


Figure 4.40 MBT/BTH/BTSA ternary metabolisation study.

4.5.1.2 Conclusions from MBT/BTH/BTSA study

It has been shown from studies on single substrate systems that each of the three components in this system are biodegradable in the order BTSA>BTH>MBT. BTSA degradation was shown to be very rapid and complete when present as sole (thiazole) substrate (Section 4.3.5) which is in contradiction to the findings of Knapp (1975), Mainprize *et al.* (1976), De Vos *et al.* (1993b), and De Wever and Verachtert (1994) who found BTSA to be non-biodegradable, although thought to be recalcitrant rather than toxic.

During treatment of BTSA the main metabolites observed were BTH and MBT with BTOH generation occurring approximately 24–48 hours after initial inoculation of the biomass with BTSA. Phenol removal was shown to be inhibited to a degree when BTSA alone was fed to the biomass; however, in this experiment, phenol removal was complete and not inhibited. The metabolite MeBTH was generated by the system within 24 hours indicating that biomethylation occurred very quickly. By contrast, when BTH is fed to the biomass as sole carbon (thiazole) source, no methylation of the thiazole ring occurred to produce the metabolite MeBTH, but rather methylation was concurrent with ring scission of the thiazole ring to yield 2-(methylthio)aniline. The appearance of MeBTH during the course of this experiment indicates that a different mechanism/enzyme substrate pathway is operating.

BTOH appears in all substrate combinations where MBT is present and when BTSA was metabolised to generate a significant yield of MBT. This provides further confirmatory evidence that BTOH features along the biodegradation pathway for MBT. However, in this experiment the biomass was stimulated in such a way that a different set of degradative pathways were observed to operate, in which the metabolite responsible for inhibition of phenol removal was not generated. The consequence of this was that no phenol was observed in the effluent during treatment of the MBT/BTH/BTSA mixture.

4.5.2 BTH/BTOH/MBT

Experimental details were exactly the same as for the previous ternary substrate study.

4.5.2.1 Results of BTH/BTOH/MBT study

Very little in the way of metabolites were produced by the biomass within the first 24 hours, the only significant metabolite being the unknown polar intermediate at t_R 3.3 mins observed in a number of the other thiazole studies. Low levels of BTH were also detected (Figure 4.41).

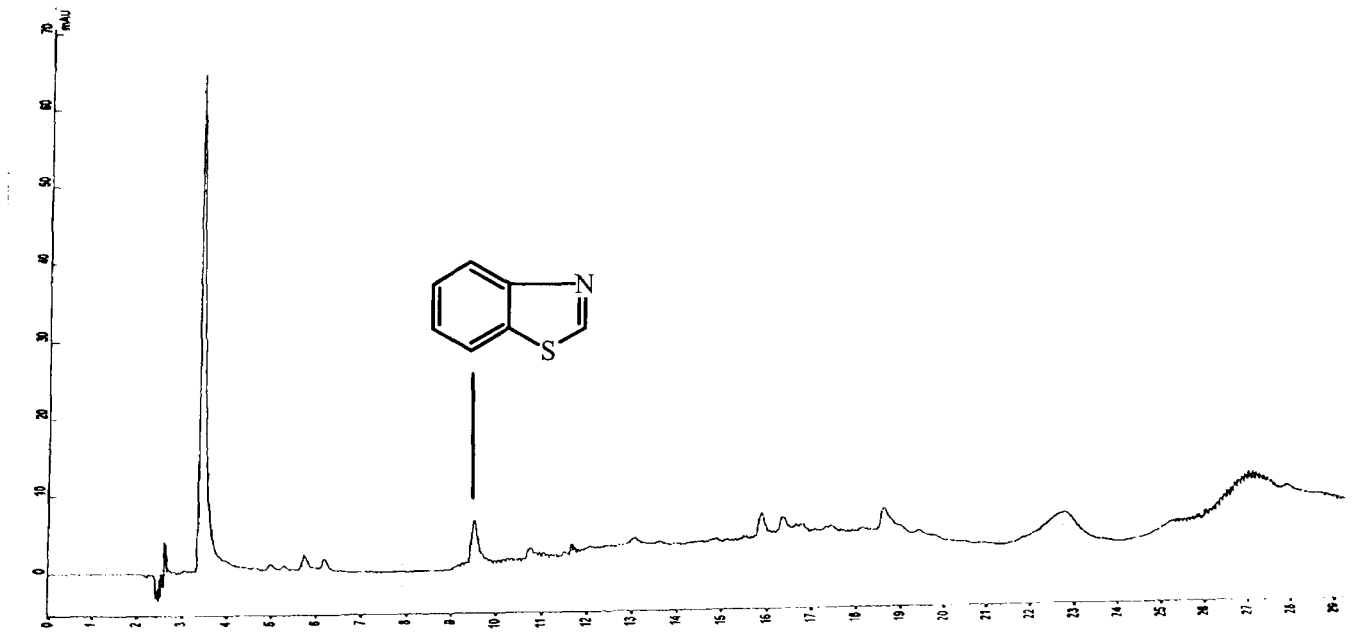


Figure 4.41 BTH/BTOH/MBT HPLC 1. The first 24 hours.

Effluent samples collected for days two and three were very similar in metabolite composition and concentration to the analysis results for the first sample. However, by the end of day four, traces of BTOH, MeMBT and MeBBT were detectable in the effluent. BTH levels continued to increase slightly whereas the concentration of the polar intermediate at t_R 3.3 mins was lower compared to the previous day's analysis.

At some time between days five and six the biomass became very active with a dramatic reduction of all species detected. This coincided with an increase in the production of the metabolite *N*-MeMBT and a sudden increase in the levels of MBT, which up to this point had not been present in the effluent from the bioreactor.

All metabolites increased during the next 24 hours which would have suggested that the biomass was experiencing a degree of inhibition. However, this effect was clearly only short-lived as evidenced by the rapid removal of all input substrates and generated metabolites. Table 4.9 gives the analytical data for this experiment and the associated chart is shown in Figure 4.42.

Table 4.9 Peak area response for BTH/BTOH/MBT metabolisation study.

Days	1	2	3	4	5	6	7	8	9	10
Polar Compd t_R 3.0 mins	69145	69368	67112	50351	21305	4282	43290	37554	21603	7321
BTH	6917	9176	14682	23827	18266	6693	21906	19063	15125	15512
2-MeMBT	0.5	0.05	0.5	13656	9672	3078	8159	6252	4413	3359
N-MeMBT	0.5	0.5	0.5	2918	4015	5256	16257	15114	11265	3980
BTOH	0.5	0.5	0.5	2504	1856	1011	0.5	0.5	0.5	0.5
MBT	0.5	0.5	0.5	0.5	0.5	0.5	58101	51011	46593	35120

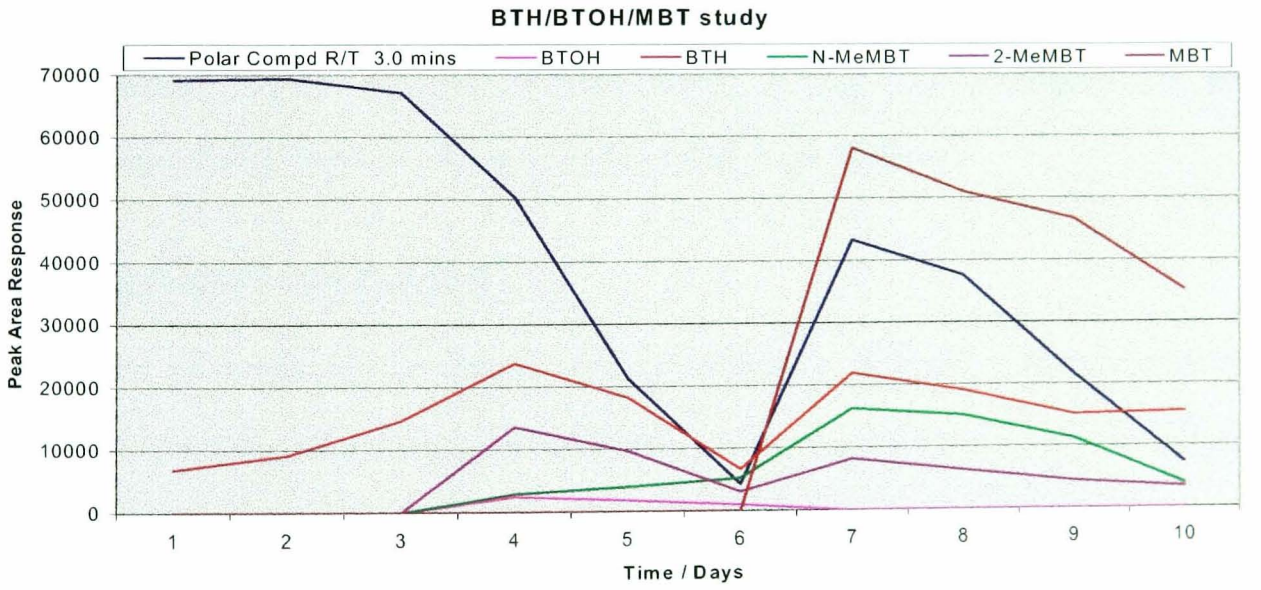


Figure 4.42 BTH/BTOH/MBT metabolisation study.

4.5.2.2 Conclusions from BTH/BTOH/MBT study

It has been shown in the earlier studies investigating the biological degradation of BTH and MBT in separate experiments, that both of these undergo a series of biotransformations which include a methylation step quite early on in the metabolic pathway. Methylation even occurs with the binary mixture MBT/BTH.

When BTOH is present either as the sole substrate or as part of a binary or ternary system the methylation processes are shown to be suppressed and only occur later on as the system has acclimated to the presence of BTOH. In the presence of BTOH, methylation is seen to occur from day four onwards, in contrast to the situation with BTOH absent when the onset of methylation is within 24–48 hours.

Degradation of BTOH again proceeded quite rapidly and without inhibition by either the BTH and/or MBT present. The results also confirmed that BTSA does not feature along the metabolic pathway involving BTH/BTOH/MBT, since none was detected during the course of the experiment. BTH degradation has been shown to proceed via the generation of a number of methylated metabolites, but under the conditions of this experiment BTH removal took place via an alternative mechanism not involving methylation.

4.6 Quaternary substrate biodegradation study

Biodegradation has been studied for single, binary and ternary substrate systems in which various combinations of MBT, BTH, BTOH, BTSA were investigated. In this final experiment a mixture of all four substrates was added to the feed at the following concentrations: BTSA 100 mg/l; MBT, BTH, BTOH each 50 mg/l. All the other experimental details were the same as the previous experiments.

4.6.1 BTH/BTOH/BTSA/MBT

The results after the first 24 hours were dramatically different from the other systems studied in that there were significant quantities of all four substrates present in the effluent along with phenol. The biodegradation of the substrates appears initially to be in the order BTOH>MBT>BTH>BTSA. In general BTOH tends to be the least affected in the initial stages of the experiment when present as part of a multiple substrate system (Figure 4.43).

Samples collected and analysed for days 2 to 4 of the experiment showed that there was a steady increase in the concentration of three out of the four substrates present in the effluent. Methylation reactions were occurring within the biomass after two days which is in contrast to other systems involving BTOH where methylation was observed only to occur after four days.

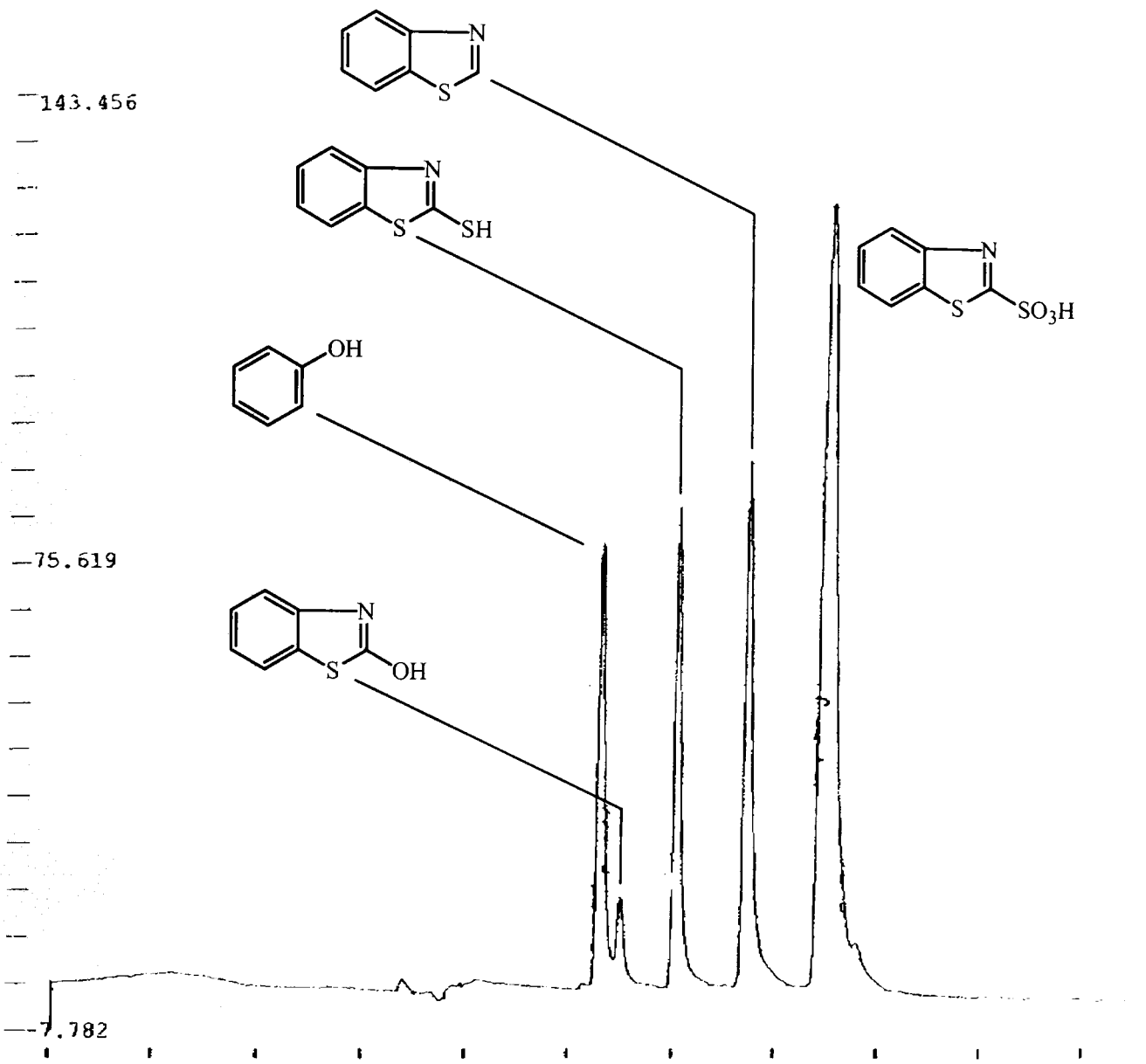


Figure 4.43 BTH/BTOH/BTSA/MBT HPLC 1. The first 24 hours.

By the end of day five BTH and MBT removal was gaining pace; 2-MeBTH production also showed signs of slowing down. BTOH removal showed signs of being inhibited indicated by an increase in the level of BTOH present in the effluent sample. Phenol removal was quite severely inhibited from day three onwards with signs of recovery occurring after eight days.

Over the remaining days of the experiment, levels of BTH dropped slightly in contrast to the steady growth in the concentration of BTOH/BTSA/MBT/2-MeBTH observed in the effluent. The levels of BTOH in particular were in contrast to the results observed in a previous experiment where BTOH removal was quite rapid and complete. In this study BTOH degradation was quite severely inhibited and to a lesser degree so was the MBT and BTSA present. The peak area response data is given in Table 4.10 and the associated chart for the quaternary metabolisation study is shown in Figure 4.44.

Table 4.10 Peak area response data for quaternary substrate study.

Days	1	2	3	4	5	6	7	8	9	10
BTSA	175452	198765	169695	177052	148762	130348	112050	121892	144922	119219
Phenol	43886	47305	62209	66440	65116	64866	64179	64475	64853	64306
BTH	65706	68334	55896	72902	69136	63056	55097	57242	59635	55131
MBT	49547	54068	73281	84358	80654	77113	59621	63205	68305	67868
BTOH	10543	25577	52650	57293	59236	61055	62677	71876	97035	82718
2-MeBTH	0.5	0.5	44722	83010	90111	84205	63648	77642	87002	89243

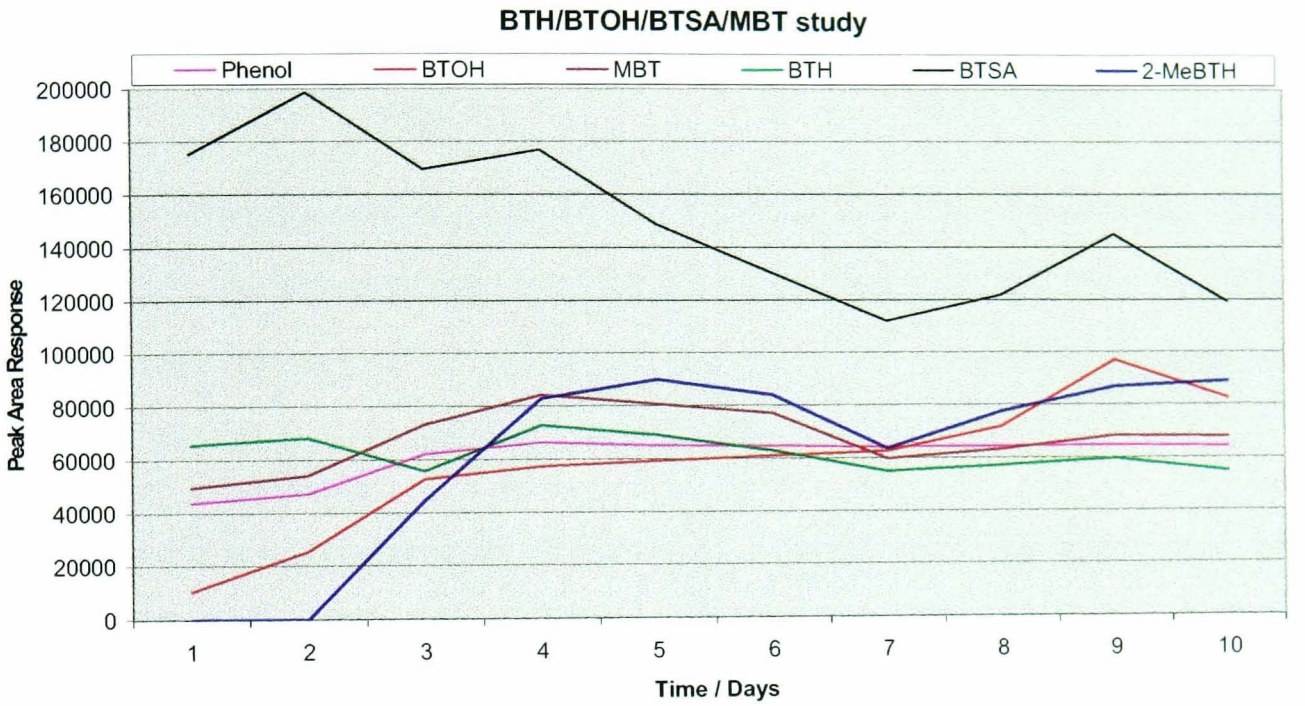


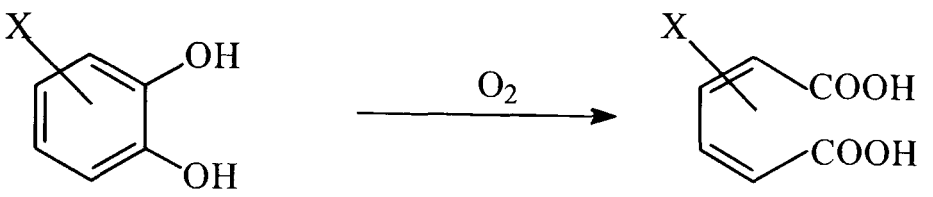
Figure 4.44 BTH/BTOH/BTSA/MBT metabolisation study.

4.6.1.1 Conclusions from BTH/BTOH/BTSA/MBT study

Clearly once the number of thiazolic compounds present reaches four with concentrations of 250 mg/l in total for the four species present in the feed to the biomass, then severe biological inhibition is to be expected. This inhibition extends to carbonaceous degradation which normally is quite resilient to inhibition shown by the high levels of phenol in the samples analysed. Furthermore, methylation processes were also suppressed in that no MeMBT analogues were detected during the experiment.

Aerobic catabolism of aromatic compounds has been studied extensively for a wide variety of microorganisms and for different natural, and a few xenobiotic, aromatic compounds (see *e.g.* Gibson and Subramanian, 1984; Haggblom and Valor, 1995). The success of a particular metabolic pathway depends on two major factors: the metabolic enzymes leading to mineralisation of an aromatic compound; and the regulatory elements (De Lorenzo and Perez-Martin, 1996).

A particular metabolic sequence used by many microorganisms for the degradation of aromatic compounds is the β-ketoadipate pathway in which ring-cleaving dioxygenases play a key role (Stanier and Ornston, 1973). Among these enzymes, catechol 1,2-dioxygenases catalyse the intradiol cleavage of catechols to *cis,cis*-muconic acids with the incorporation of molecular oxygen.



Dioxygenases incorporate both atoms of the oxygen molecule into the substrate. There are two major types of dioxygenases: one type has a requirement for NADH or NADPH and these enzymes hydroxylate the substrates, the other has no specific requirement for NAD(P)H, and functions to cleave the aromatic ring.

It is known that at high concentrations MBT is toxic to microflora (De Wever *et al.*, 1994). In studies where the MBT concentration was 50 mg/l, the initial step was biomethylation followed by hydroxylation to yield the –SOH and –OH products, the production of which was more than likely to have been mediated by the ring-hydroxylating dioxygenases. However, at higher concentrations (50–100 mg/l), this enzymatic pathway is suppressed in favour of oxidative dimerisation of the MBT to produce MBTS. This may be a protective step by the biomass to prevent build up of toxic levels of MBT, which would result in loss of biological activity. The generated MBTS is initially adsorbed on to the floc surface. The hydroxylation step then follows bond cleavage of the S–S to generate the products –SOH and $–SO_2H$ respectively.

The presence of high levels of MBT (*ca* 50–100 mg/l) and BTH/BTOH mixtures appear to suppress the ring-cleavage dioxygenases, with the result that biomethylation is observed as the initial degradation step in the pathway, rather than ring scission. In contrast, when one of MBT, BTH or BTOH are present as a sole carbon substrate the ring-cleaving dioxygenases control the pathway to yield the ring-cleaved substituted phenylamine.

Aerobic microorganisms use diverse biochemical reactions to initiate the biodegradation of nitroaromatic compounds. Reactions that attack the nitro substituents are grouped into two general categories, oxidative or reductive (Rieger and Knackmuss, 1995). With mono- or dinitro substituted aromatic compounds, the preferred route for the initial degradation is hydroxylation carried out by mono- or dioxygenases. The reaction normally results in replacement of the nitro group by an –OH group, with release of nitrite.

The results obtained in these studies of the biodegradation of a range of thiazoles are in accord with the above pathway: in the case of MBT, BTH and BTOH the nitroaromatic metabolite is biotransformed via an oxidative step with replacement of the $-NH_2$ group by an –OH group.

A common feature of the biodegradation pathway of thiazoles is biomethylation followed by ring scission. Methylation appears to be suppressed in the presence of the –OH group as shown by the lack of methylated metabolites generated during BTOH degradation. A similar situation arises where hydroxylation precedes ring scission. Hydroxylation of the hetero carbon atom of the thiazole ring precludes methylation at that carbon atom, as seen in the pathway for BTOH and in the case with BTH/BTOH mixtures.

The unknown intermediate eluting at t_R 3.3 mins in a number of the chromatograms has unfortunately not been identified during the course of this work; however, Figure 4.45 shows which conditions favour its formation and which suppress it. It can be deduced from Figure 4.45 that the MBT/BTSA combination of substrates exerts a combinative blocker/inhibitive effect on the biomass and prevents the formation of the unknown polar intermediate occurring at t_R 3.3 mins in multiple substrate systems. As yet no firm identification of this polar compound has been made, but its generation appears to be inhibited by MBT/BTSA.

The compound BTSA has long been linked to MBT degradation. However, the findings of this work contradict this view. No BTSA was detected during biodegradation of MBT, BTH, or BTOH, though BTSA was a metabolite when MBS was degraded.

Single substrate studies	
MBT	✗
BTH	?
BTOH	Trace
BTSA	✓

Binary substrate studies	MBT	BTH	BTSA	BTOH
MBT	✗	✓	✗	Trace
BTH	✓	?	?	✓
BTSA	✗	?	✓	✓
BTOH	Trace	✓	✓	Trace

Ternary substrate studies	
BTH/BTOH/MBT	✓
MBT/BTH/BTSA	✗

Quaternary substrate study	
BTOH/BTH/BTSA/MBT	✗

Key
✗ = No polar intermediate at t_R 3.3 mins detected
✓ = Significant amount of polar intermediate at t_R 3.3 mins detected
Trace = Trace amount of polar intermediate at t_R 3.3 mins detected

Figure 4.45 Unknown polar intermediate generation matrix.

No methyl derivatives of MBT or BTH were observed during MBS degradation. Furthermore, no BTH was detected from the MBT generated during MBS breakdown by the biomass used in this study. If BTH had been generated it would have led to the production of BTOH. The lack of any BTOH provides further supporting evidence for the proposed pathways.

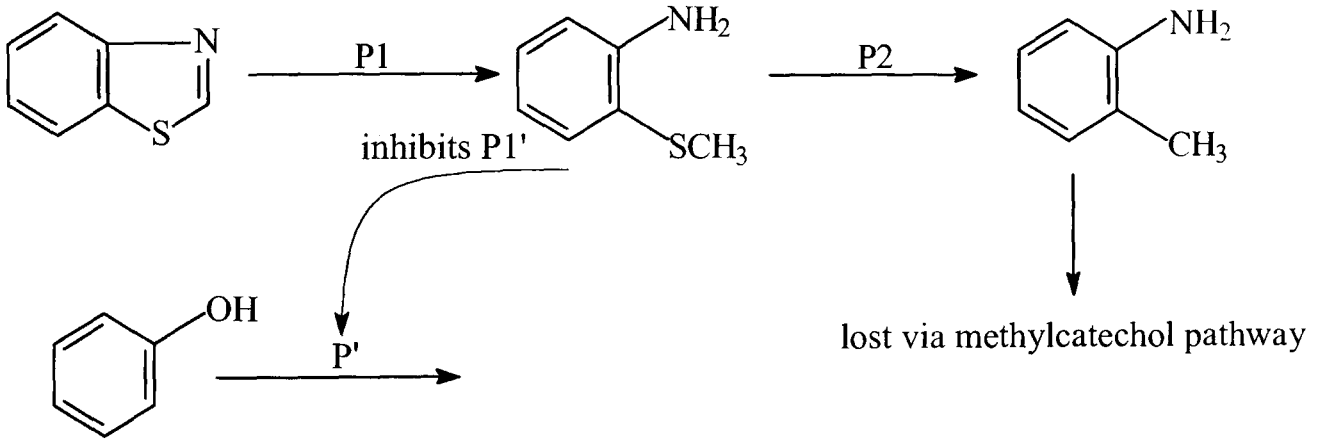
Only very low concentrations of the methylated –SO and $-SO_2$ analogues were detected indicating that, although the S–N bond was oxidised quite quickly, this was not the main pathway. The main pathway appears to involve MBT formation with the liberation of morpholine.

In previous investigations, the presence of MBT in the feed has been linked to BTSA formation (Knapp *et al.,* 1982; Drotar *et al.,* 1987; Wilson *et al.*, 1991; Brownlee *et al.,* 1992; Gaja and Knapp, 1997). This has been shown not to be the case with the biomass used in this study. In studies with MBT as sole source of organic carbon, nitrogen and sulphur, no BTSA was formed during biological removal of MBT. Furthermore, contrary to some studies (Wilson *et al.,* 1991; De Vos *et al.,* 1993), no BTSA was observed even when BTH and BTOH were introduced as additional substrates. Only with MBS as sole source of organic sulphur and nitrogen was BTSA generated by the biomass. When it is formed, removal of BTSA has been shown to take place, though very slowly especially in the place of MBT and BTH.

What is clearly apparent from the results of all the studies is that biological activity, and in particular specific enzymatic pathways, are affected not only by the incoming substrate but also the generated metabolites. For example, consider phenol removal by the specific biomass used in this study.

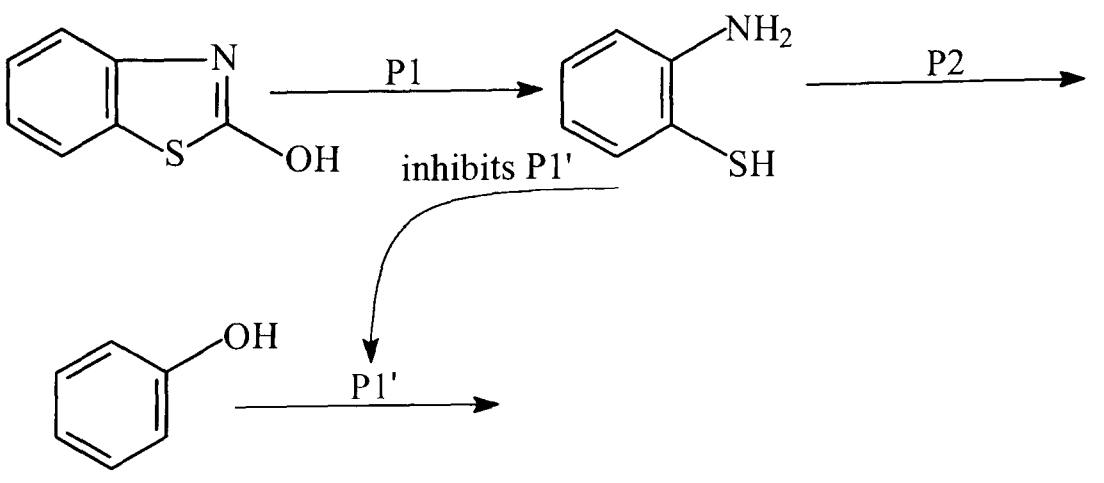
In single substrate systems, *i.e.*, BTH, BTOH, MBT and BTSA at 50 mg/l, in all cases phenol removal is inhibited leading to its observation in the effluent from the biological reactor. However, when MBT is introduced into the biomass at 100 mg/l, a different removal pathway for MBT operates in which the phenol removal pathway is not inhibited since no phenol is observed in the effluent.

Consider first the abbreviated pathway for benzothiazole (BTH) degradation at 50 mg/l.



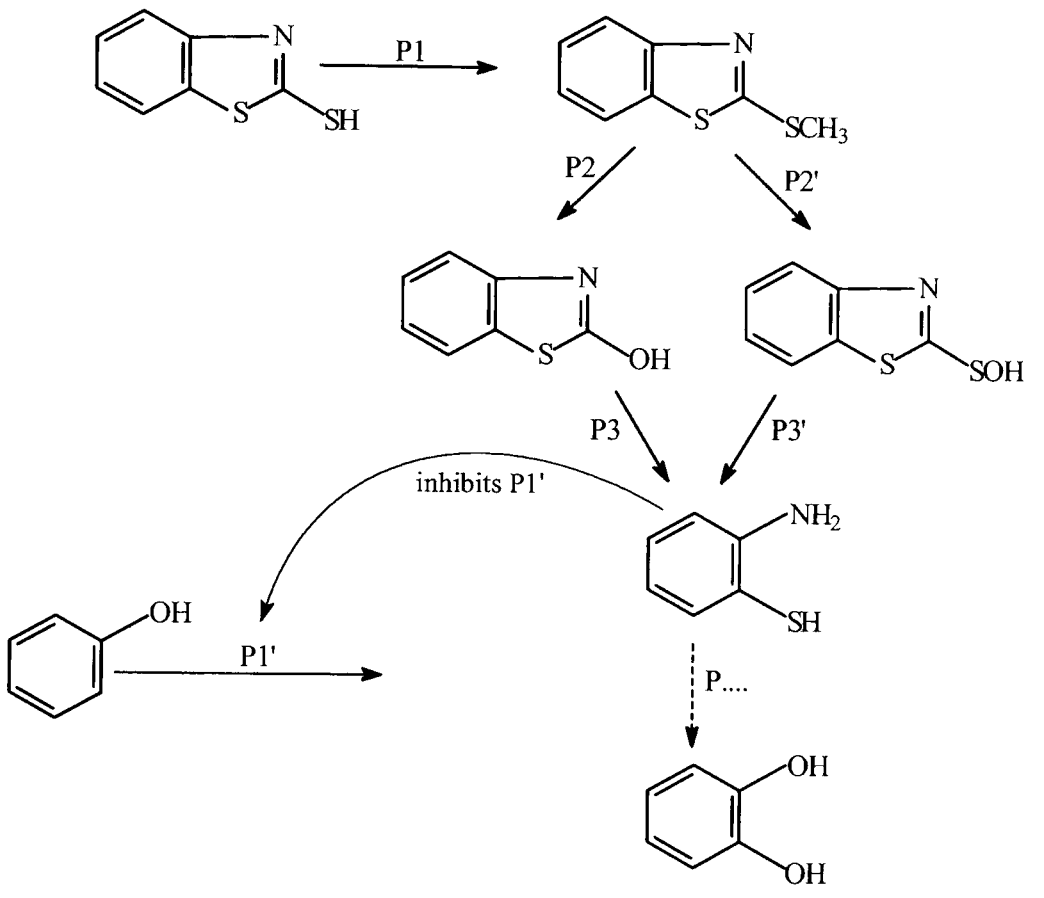
In this example early production of the metabolite 2-(methylmercapto)aniline from the BTH P1 pathway inhibits the P1′ pathway, resulting in the appearance of phenol in the effluent from day one.

Next, consider the abbreviated pathway for 2-hydroxybenzothiazole (BTOH) degradation at 50 mg/l.

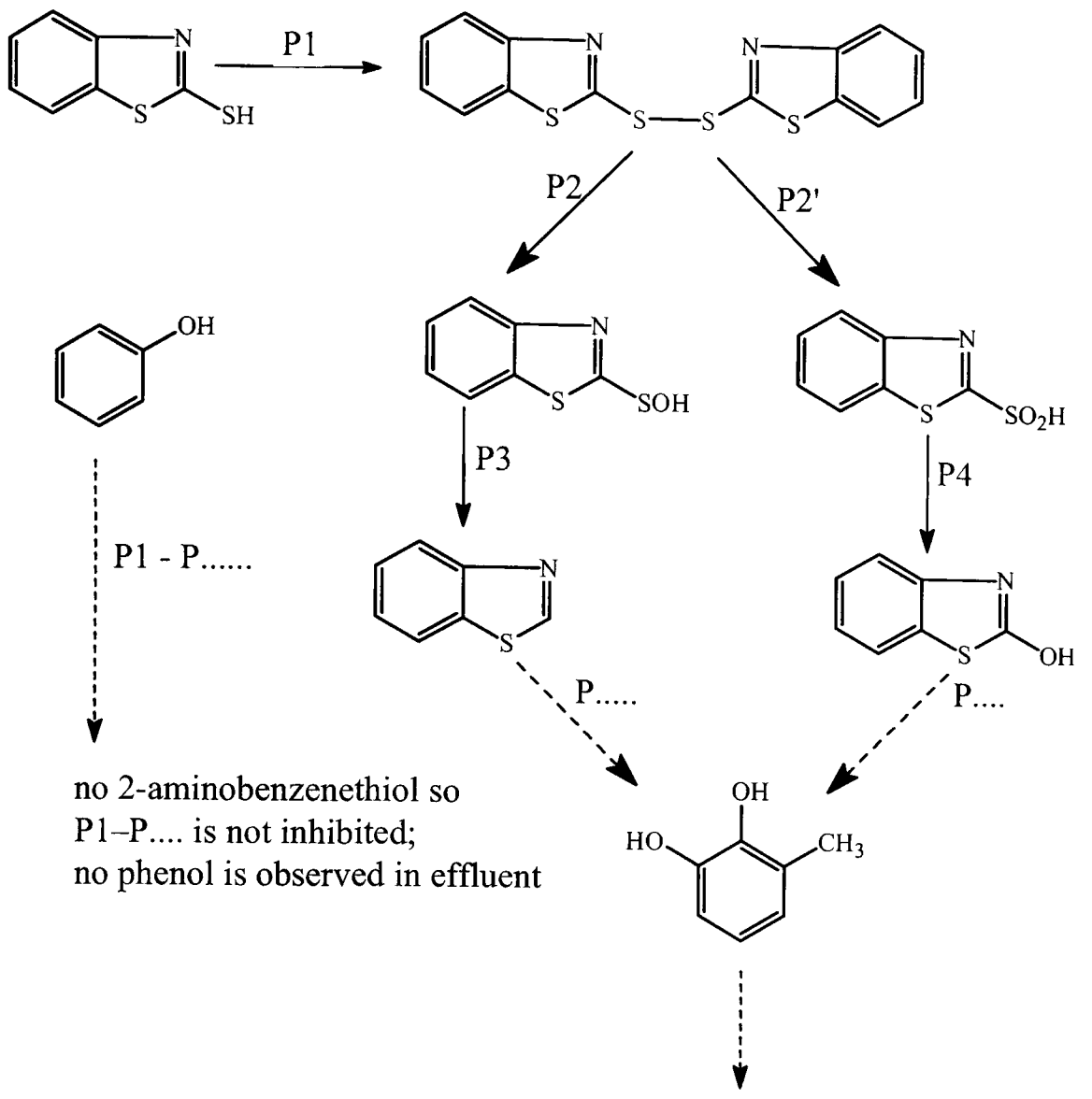


Initial ring cleavage to produce 2-aminobenzenethiol appears to occur very early in the BTOH degradation pathway in a like manner to the pathway for BTH degradation above. As in the case with BTH, phenol was observed in the effluent from day one of the experiment indicating that. its removal was inhibited.

However, with mercaptobenzothiazole (MBT) at 50 mg/l, phenol was not detected in the effluent until the end of day three of the experiment. This coincided with the production of 2-aminobenzenethiol, which in turn begins to inhibit the phenol removal pathway.



However, when MBT was fed to the biomass at 100 mg/l then a different set of reactions apparently occurred since phenol was not observed in the effluent. This implies that different enzymatic mechanisms were taking place. In the proposed scheme shown below, no 2-aminobenzenethiol is generated to account for the observation that the phenol metabolisation pathway was not inhibited.



The above examples also demonstrate how the various pathways are stimulated by the presence of the parent substrate and the daughter metabolites generated by the biomass during biological breakdown.

A clear example of this was shown by the treatment of MBT at two different concentrations. At 50 mg/l, MBT was only slowly degraded with the generation of MeMBT and BTOH and further along the pathway 2-aminobenzenethiol. However, when the MBT concentration was increased to 100 mg/l a different pathway was stimulated with dimerisation of MBT to generate MBTS. In this scheme, both BTH and BTOH are metabolites along the degradation pathway for MBTS; in contrast no BTH is observed with 50 mg/l MBT. Furthermore, it has been shown that BTH is a precursor to the two species 2-aminobenzenethiol and 2-(methylmercapto)aniline, providing further supporting evidence that different pathways exist for each specific substrate.

4.7 Memory effect of the biomass

The manufacturing plant required information on the memory effect of the biomass in order to investigate possible batch production of thiazoles at the Ruabon site. The plan was to follow a so-called 'campaign' manufacturing regime: following a period of production activity the plant(s) would be shut down for up to three months at a time. This required the bioreactors to be able to cope with the expected effluent on a 'stop-start' basis.

To investigate this memory phenomenon the reactor R2 was fed a mixture consisting of all the expected components from the Ruabon production site with the exception of the thiazolic components. The initial experiment was carried out over a three week period at the end of which the normal 'Vitox' feed was reinstated to the bioreactor and the discharge analysed over the next 24 hours for any significant changes. In particular the ammonia and TOC levels were measured to ensure compliance with discharge consents.

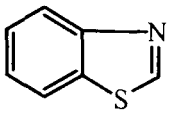
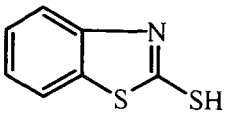
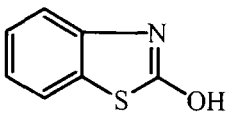
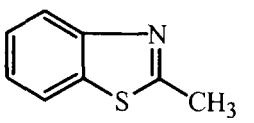
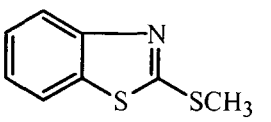
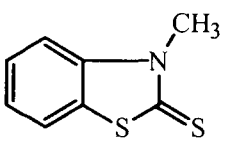
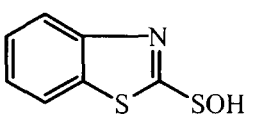
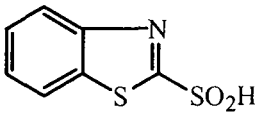
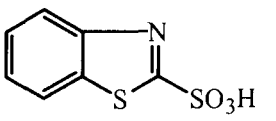
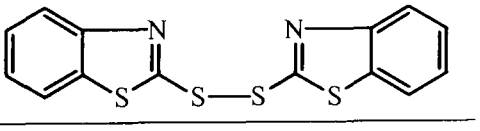
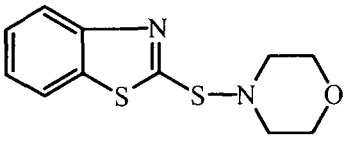
The results indicated that the biomass was not adversely affected by the removal of thiazolic compounds for the short period of three weeks, confirmed by the insignificant change in residual ammonia and TOC on reinstatement of the thiazoles to the bioreactor. A slight increase in the TOC was observed for 12 hours, peaking at 28 mg/l compared with a daily average of 27 mg/l. The experiment was repeated but for a period of six weeks, results from this second experiment were almost identical to those from the first experiment in that the TOC was observed to increase for a short period but this time it remained within the compliance limit of 30 mg/l.

The procedure was carried out on the main waste water treatment plant in the same way as in the laboratory, i.e. first a three week experiment followed by a longer six week run. The results from the main plant tracked those from the laboratory in that a short period of high TOC was observed after the first experiment and similarly in the second, but still remaining within the consent limit imposed by the Environment Agency.

The above results demonstrate that the biomass retains a memory over a short period of circa six weeks and is able to quickly recover and begin biological activity and treat incoming substrates within a 24 hour period following re-exposure to the absent substrates.

Appendix 4A

Full structures, abbreviations and nomenclature of the benzothiazoles studied.

Benzothiazole (BTH)	
2-Mercaptobenzothiazole (MBT)	
2-Hydroxybenzothiazole (BTOH)	
2-Methylbenzothiazole (MeBTH)	
2-Methylmercaptobenzothiazole (MeMBT)	
3-Methylbenzothiazole-2-thione (MeBBT)	
Benzothiazole-2-sulphenic acid (BTSOH)	
Benzothiazole-2-sulphinic acid ($BTSO_2H$)	
Benzothiazole-2-sulphonic acid (BTSA)	
Dibenzothiazole-2,2′-disulphide (MBTS)	
2-(4-Morpholinothio)benzothiazole (MBS)	

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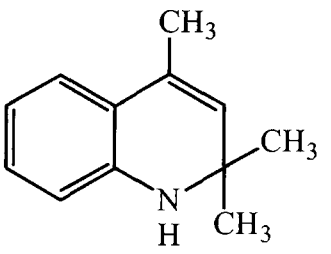
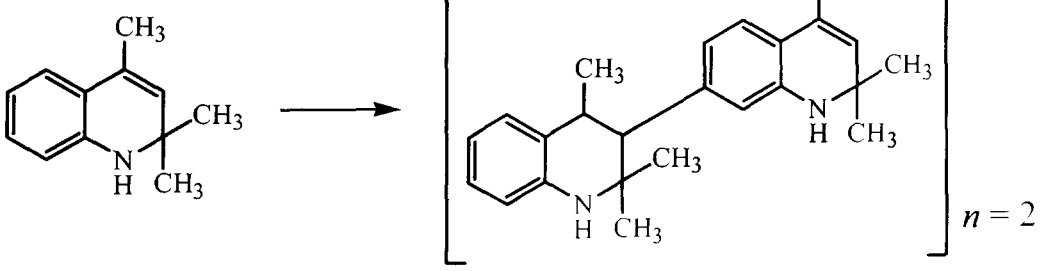
CHAPTER 5
BIOLOGICAL DEGRADATION OF NON-THIAZOLIC COMPOUNDS

5.1 Biodegradation of 1,2-dihydro-2,2,4-trimethylquinoline

The compound 1,2-dihydro-2,2,4-trimethylquinoline, known as Flectol A, is the monomer of the product known commercially as Flectol TMQ. The product is a polymerised mixture consisting of a number of oligomers over the range $n = 1$ to $n = 6$. The principal component of the polymerised product is the dimer, which constitutes 30–35% of the overall oligomer mixture. The monomer is the only water-soluble fraction and constitutes less than 1% of the commercial product. However several process streams contain much higher concentrations. Where these process streams are in contact with waste stream, the monomer can reach concentrations of up to 0.5% in the effluent feed to the water treatment plant. Once in the storage tank the insoluble fraction settles out and the monomer being the only water soluble fraction slowly dissolves over time. Flectol A has the structure shown and is polymerised to produce a mixture of oligomers with structures typified by the dimer.



Flectol A



Flectol monomer

Flectol dimer

A sample of the monomer was purified by distillation to produce a colourless product, which by GCMS was shown to be 99.5% pure. This material was then used to investigate the biodegradability of Flectol A using a mixed culture aerobic activated sludge system. The monomer was spiked into the synthetic feed (the same base matrix as used for the

study of thiazolic compounds described in Chapter 4) at 50 mg/l and fed onto the biomass of reactor R3 over a twelve day period. Samples were collected at the end of each 24 hour period and analysed by GCMS for residual monomer and associated metabolites.

5.1.1 Results of Flectol A degradation study

A sample of effluent was taken from reactor R3 prior to the introduction of the Flectol monomer. Subsequent GCMS analysis confirmed the system to be free of any monomer or associated components.

Analysis by GCMS of the sample collected after the first 24 hours following introduction of the monomer indicated that the monomer was present but expected metabolites were either below the detection limit or not present (Figure 5.1)

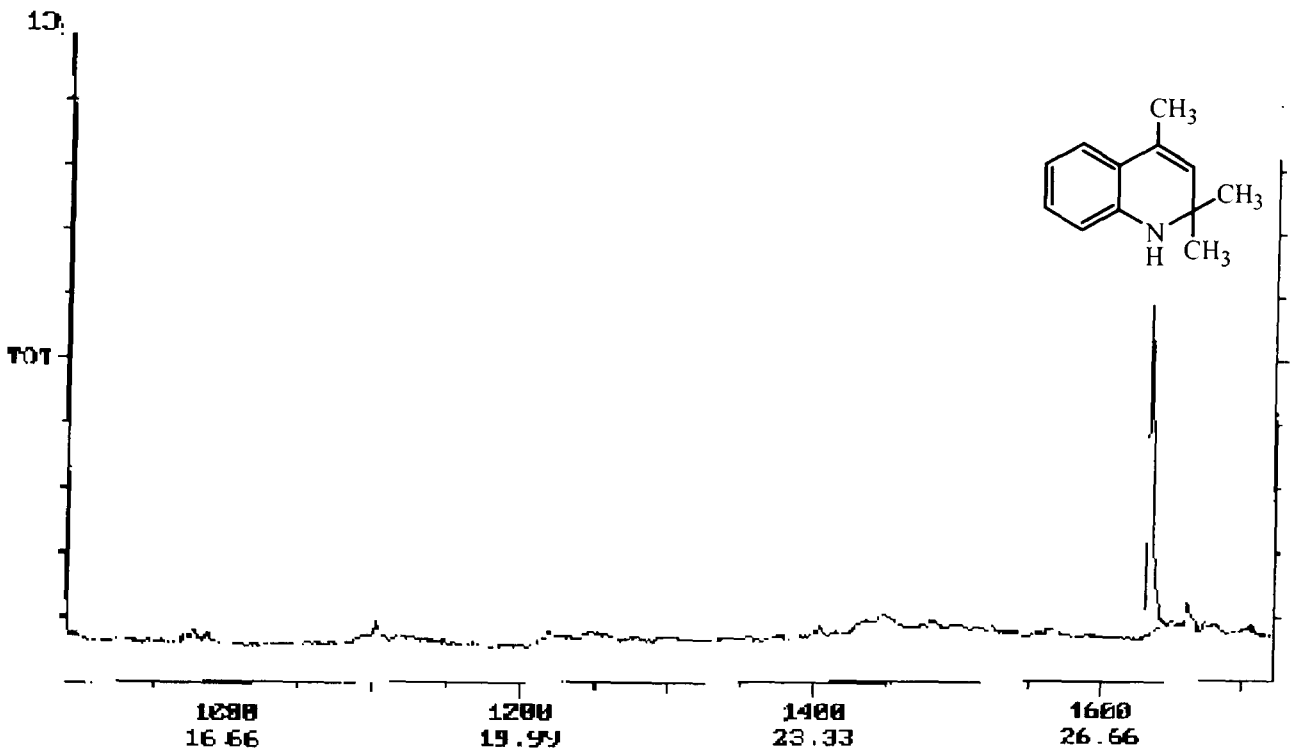


Figure 5.1 GCMS TMQ 1. The first 24 hours.

Samples collected over the following days confirmed that biological degradation of the monomer was taking place. However, the levels of the monomer continued to increase to a maximum, as did associated metabolites. Figure 5.2 shows a typical chromatogram obtained from analysis of the effluent collected during the experiment.

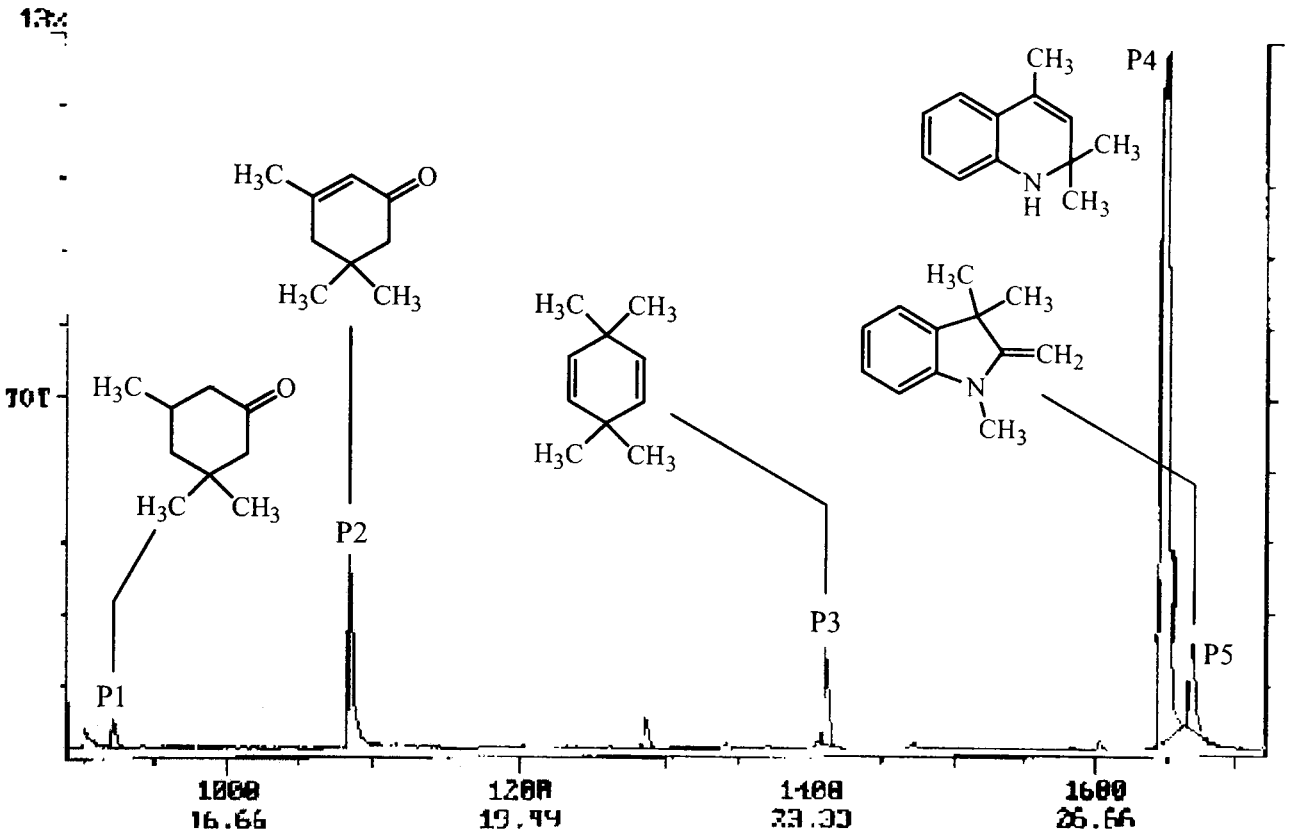


Figure 5.2 GCMS TMQ2.

The results of the chromatographic analyses are given in Table 5.1; each component is expressed in terms of peak response. The results are also presented in chart form in Figure 5.3 showing trends observed for each component.

Table 5.1 Analysis results of monomer biodegradation studies.

time/days	P1 response	P2 response	P3 response	P4 response	P5 response
1	0	0	0	0	0
2	0	0	0	88410	0
3	82545	729642	279167	12985168	349844
4	79237	703971	263464	13182412	324940
5	254228	1910490	753865	40441348	3546890
6	341170	2288916	871687	58957099	2523724
7	449976	3247901	1183664	86595503	3524948
8	428827	4017404	1360086	103748934	4513954
9	0	6341340	1590072	136567416	6076414
10	0	31241577	9026052	391650783	33712598
11	0	6234458	2116607	145447501	6012269
12	0	316708	251655	34197803	662011
13	0	0	0	14746322	591469

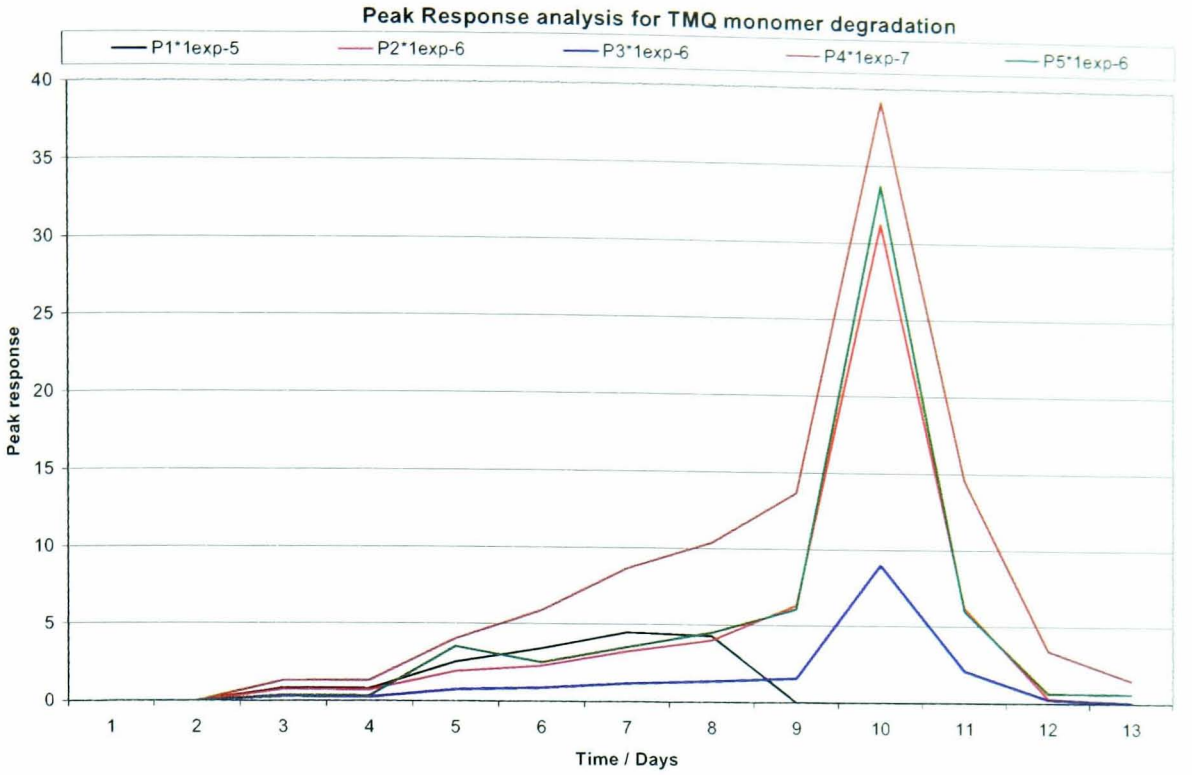
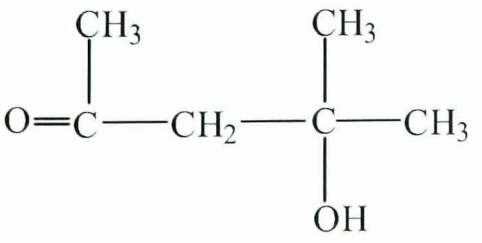


Figure 5.3 Analysis trend for Flectol TMQ monomer degradation.

The various metabolites detected were identified on the basis of their mass spectra. Four major metabolites were identified and are described below (see Figures 5.4 to 5.8 for mass spectra and structure of identified metabolites). The species P1 would appear to have an inhibitory effect on the biomass and slows down the biotransformation of the monomer and subsequent metabolites. Once the species P1 is no longer present or being generated by the biomass the other metabolites P2, P3 and P5 are rapidly generated by the biomass along with the monomer (P4).

A minor and transient metabolite observed both in the laboratory biological reactor and the main water treatment plant was identified as 4-hydroxy-4-methyl-2-pentanone.



4-hydroxy-4-methyl-2-pentanone

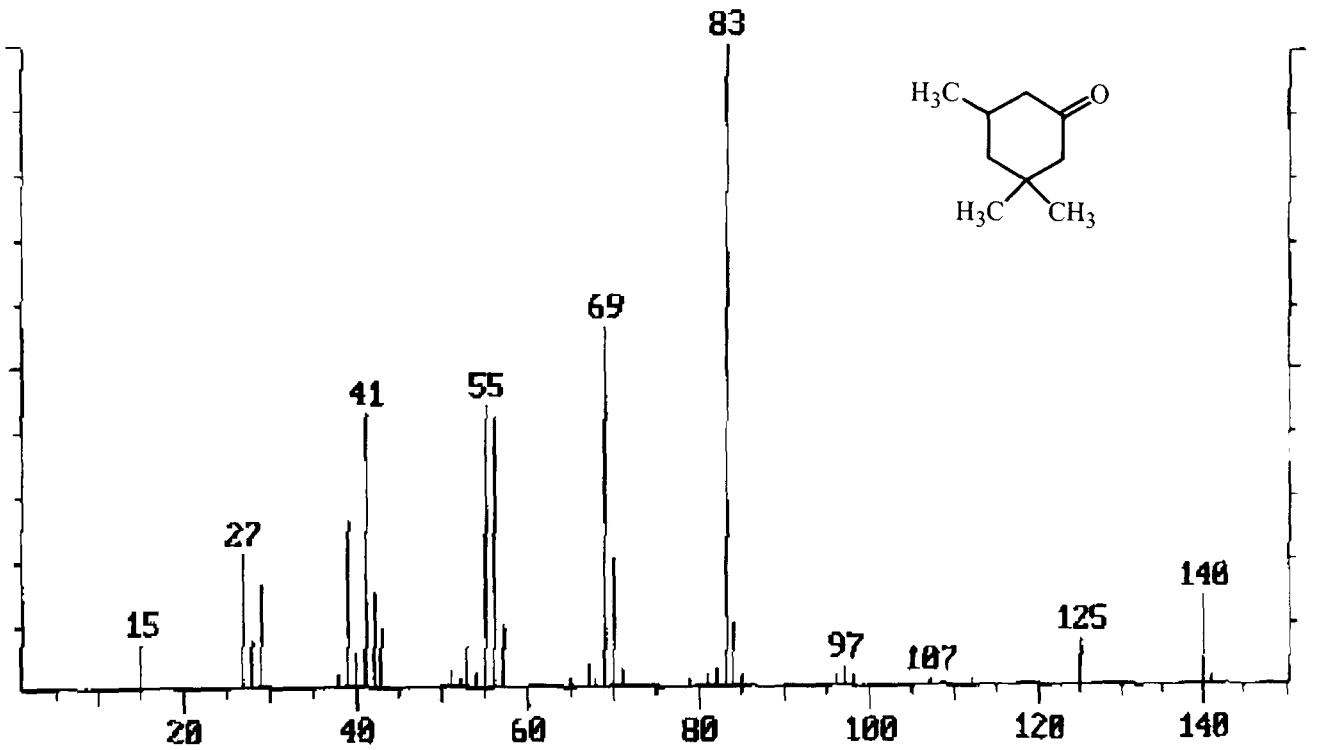


Figure 5.4 P1 3,3,5-trimethylcyclohexanone.

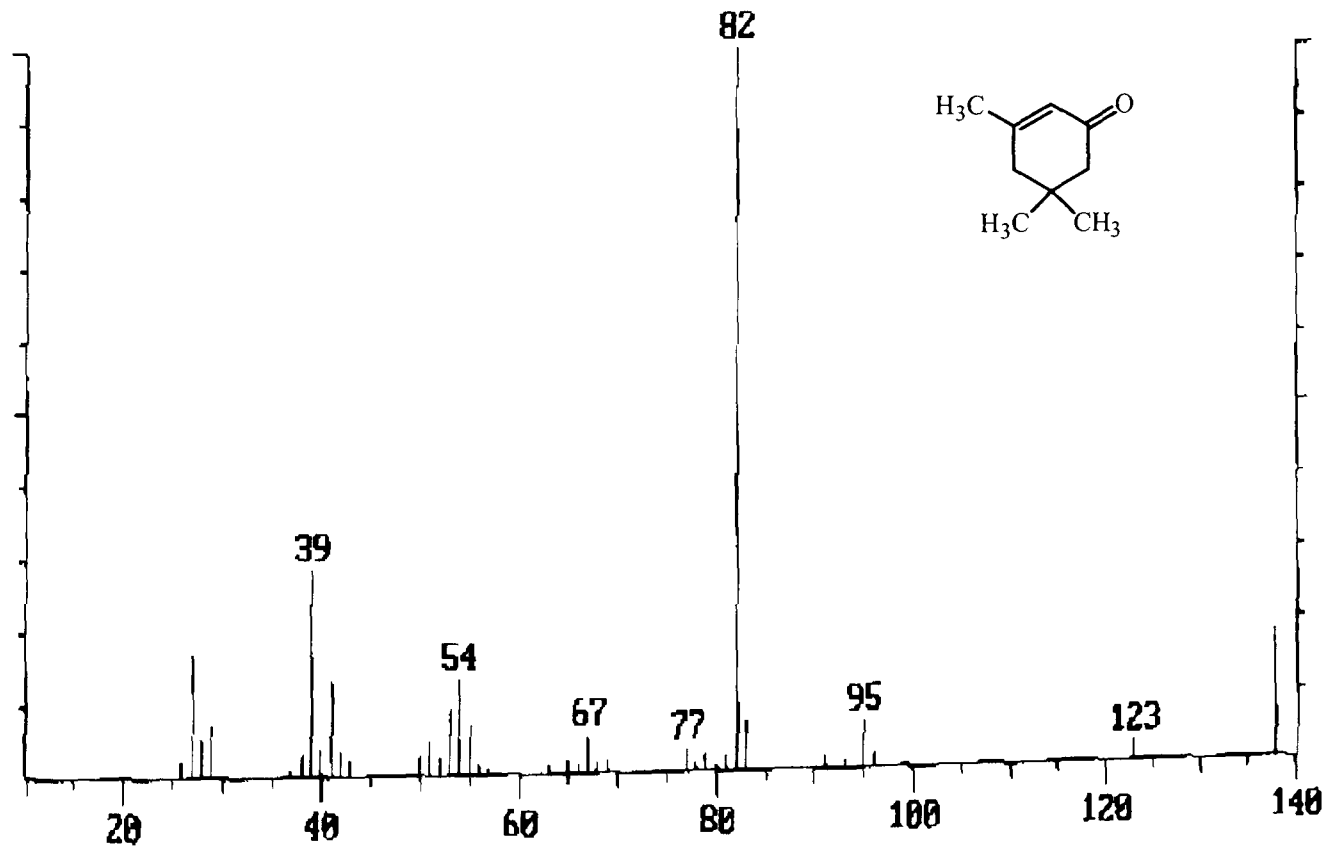


Figure 5.5 P2 3,5,5-trimethylcyclohex-2-ene-1-one.

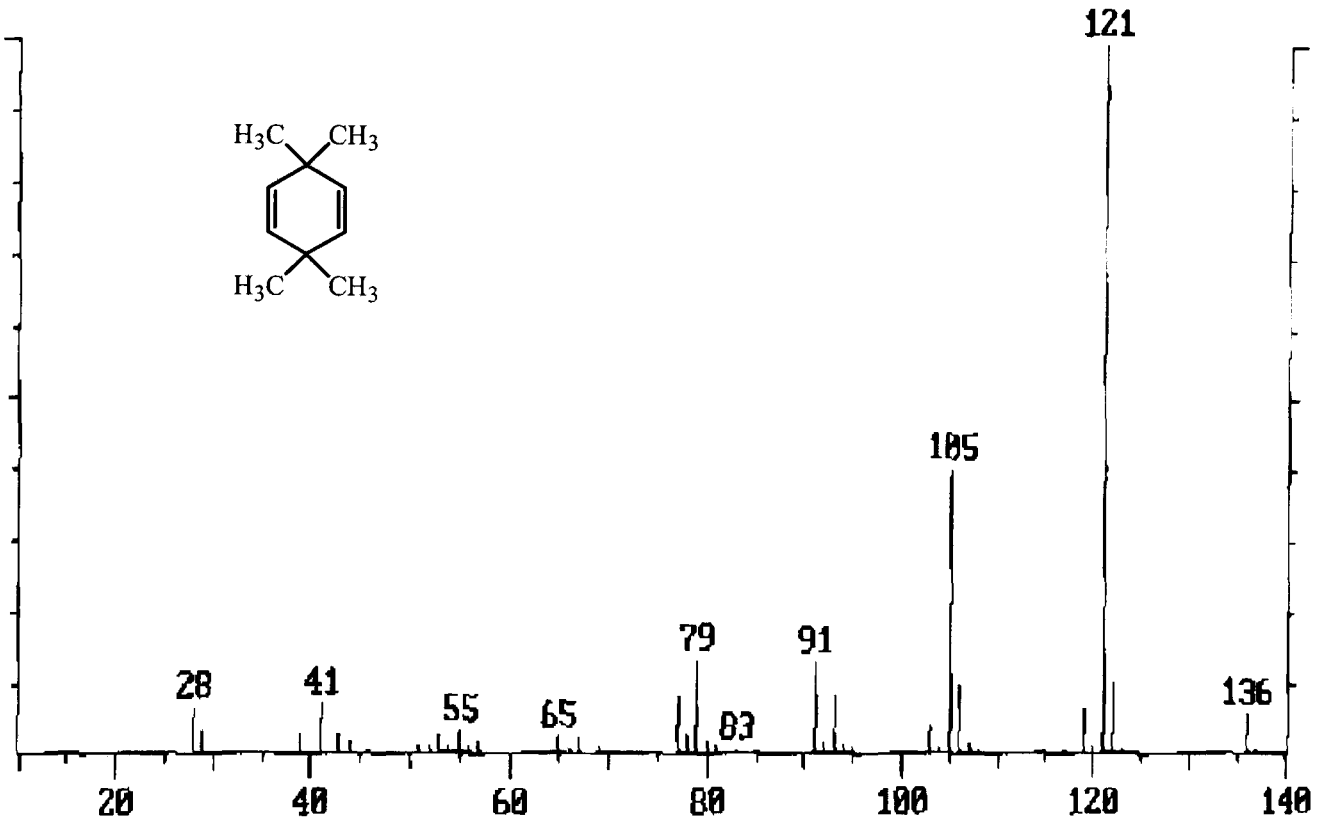


Figure 5.6 P3 3,3,6,6-tetramethylcyclohexa-1,4-diene.

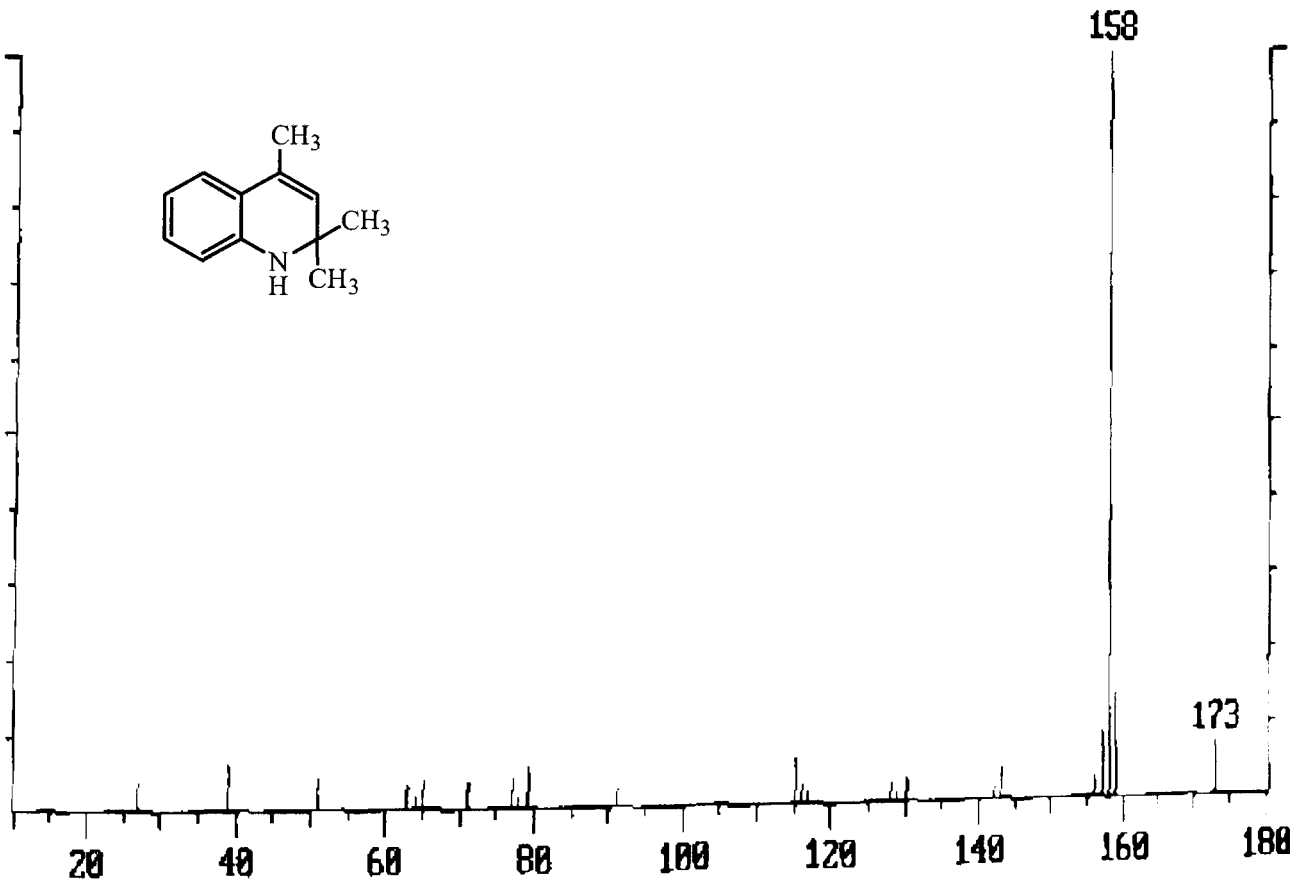


Figure 5.7 P4 1,2-dihydro-2,2,4-trimethylquinoline (monomer, not a metabolite).

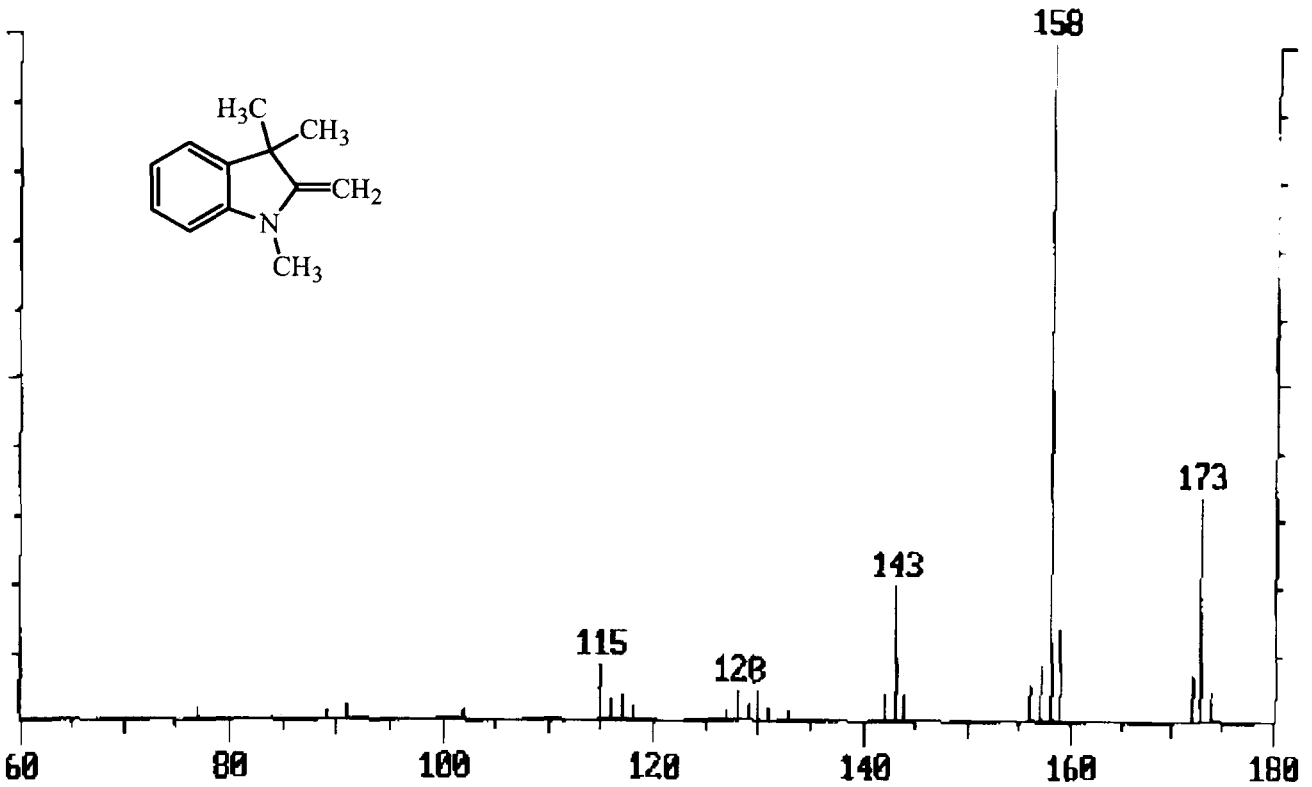


Figure 5.8 P5 2-Methylene-1,3,3-trimethylindoline.

5.1.2 Discussion of Flectol A degradation study

As far as can be determined, this is the first report of aerobic degradation of 1,2-dihydro-2,2,4-trimethylquinoline by a mixed culture activated sludge. The results of this study demonstrate that the monomer is biodegradable, albeit after a suitable acclimation period, with the production of biodegradable metabolites. It would appear that the heterocyclic ring is initially cleaved to produce an oxidised moiety followed by a series of methylated species.

The species identified as P1 is shown to be the component that is the most readily biodegradable of the various metabolites generated. This component may be a precursor to the component labelled as P3; the presence of P1 may also inhibit the production of P3, hence the sudden appearance of the latter following the removal of P1 from the system, *i.e.*, the product of pathway Flectol TMQ $\longrightarrow$ P1 inhibits the production of species P3 from TMQ (Figure 5.9).

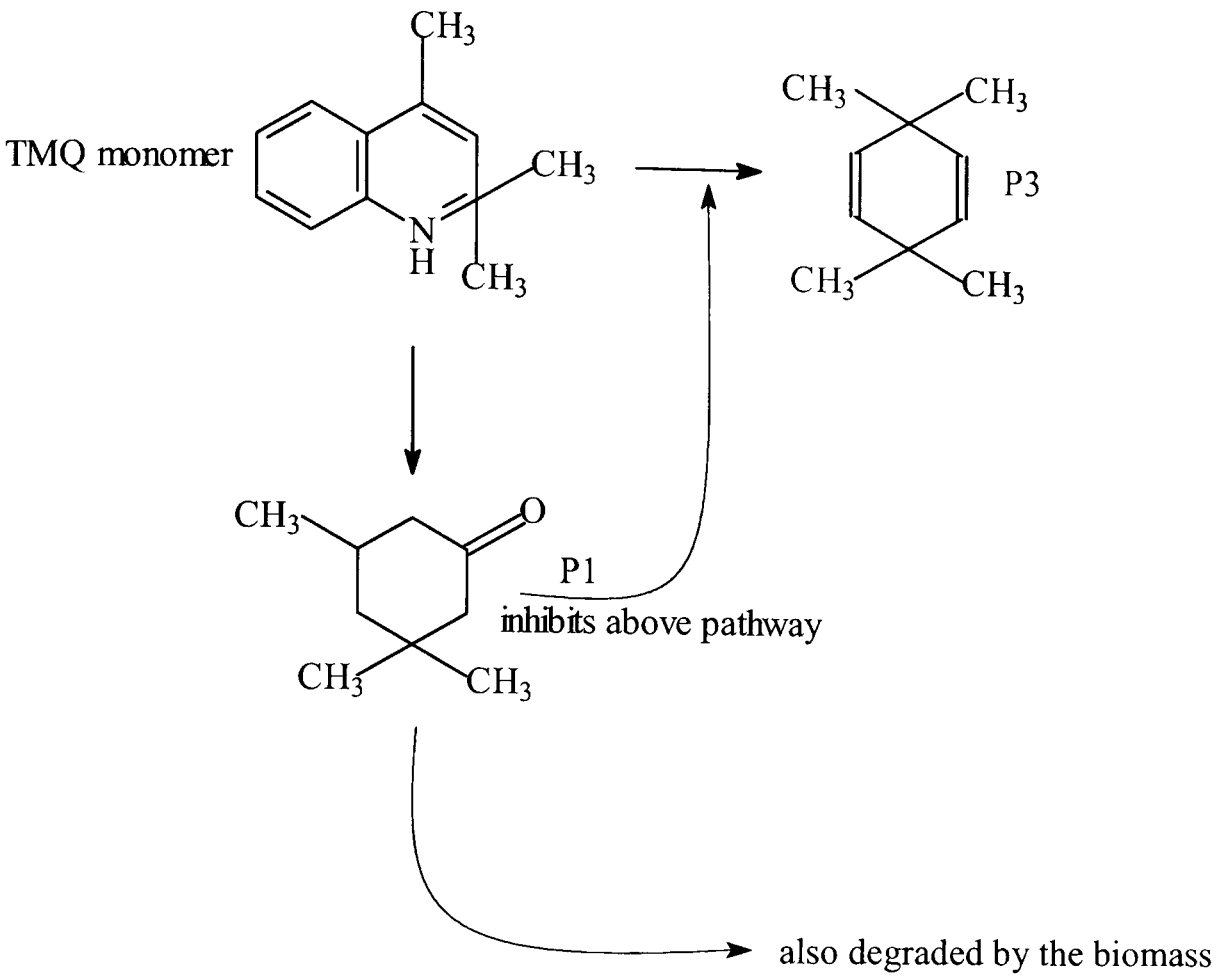


Figure 5.9 Proposed metabolic routes for degradation of Flectol A.

The 4-hydroxy-4-methyl-2-pentanone (4HMP) appears to be a product of ring scission yielding aniline via the proposed hypothetical pathway from TMQ monomer to 4HMP shown in Figure 5.10, overleaf.

The only supporting evidence for the above hypothesis is the presence of trace levels of both aniline and 4-hydroxy-4-methyl-2-pentanone, both occurring at the same time and also only transiently during the experiment. This is also analogous to the process chemistry for the manufacture of the polymerised Flectol TMQ.

As stated above, this appears to be the first report of aerobic biodegradation of the Flectol TMQ monomer by a mixed consortia of activated sludge. However, there are two recent reports detailing studies of the biodegradation of other *N*-heterocyclic compounds by Jianlong *et al.* (2001) and Yongmei *et al.* (2001). These two papers report on the biodegradation of indole, quinoline and *iso*quinoline along with methylated analogues.

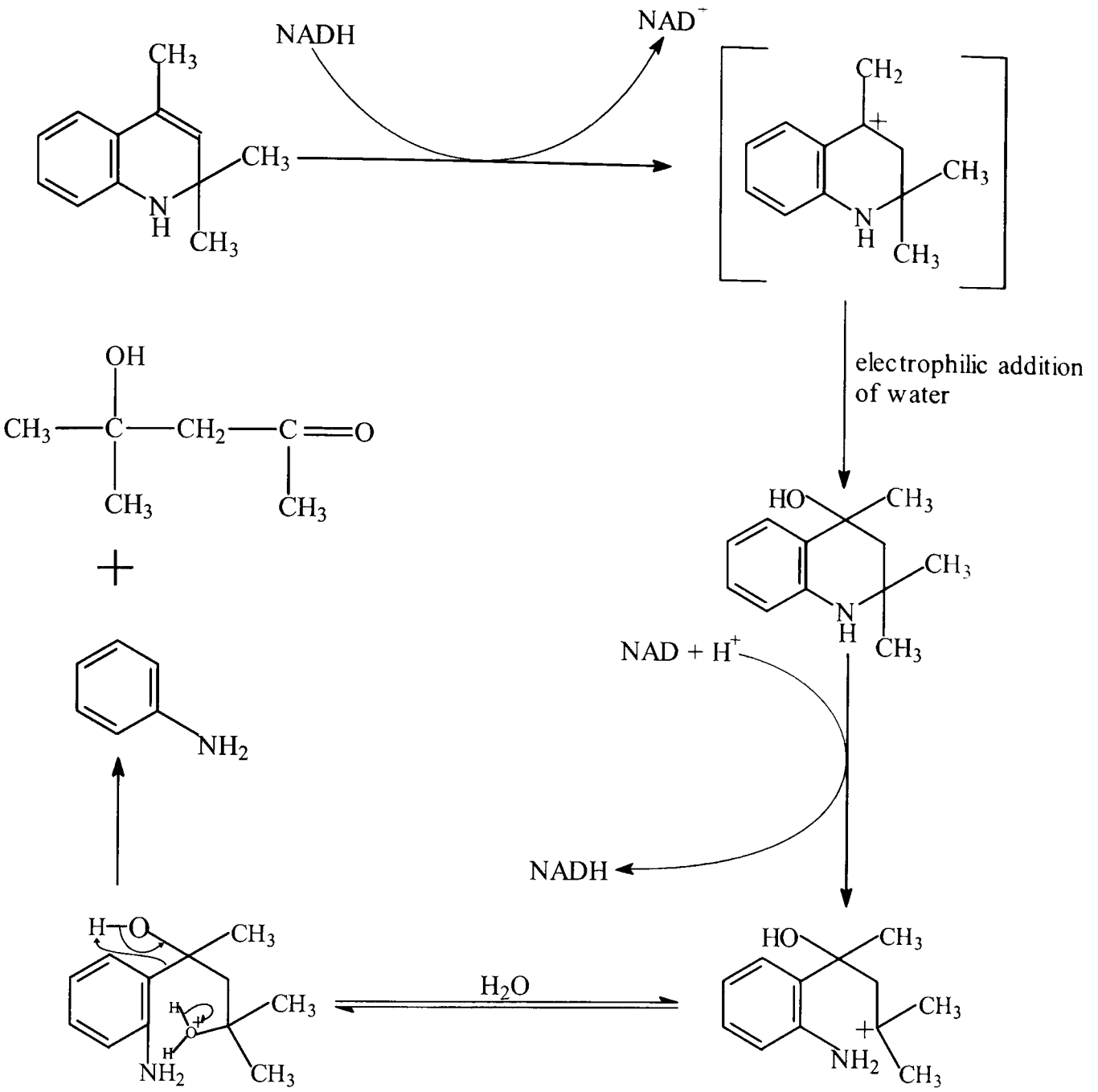
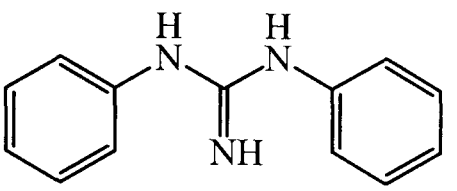


Figure 5.10 Proposed metabolic routes for degradation of Flectol A.

The findings of Yongmei *et al.* (2001) were that compounds such as pyridine, indole, quinoline, *iso*quinoline and 2-methylquinoline were effectively degraded by denitrifiers in acclimated activated sludge within 60 hours. They proposed that the order of ease of degradation was pyridine > indole > quinoline > 2-methylquinoline > *iso*quinoline, which was mainly dependent on their chemical structure. This hypothesis would support the findings of this work investigating TMQ monomer degradation and associated metabolites. The species P1 represents the simplest structure of the various metabolites identified and is degraded the quickest. The monomer (P5) conversely represents the most complex molecule of the group under investigation and is shown to be the more persistent compound under the conditions of the experiment.

5.2 Biodegradation of diphenylguanidine

The compound diphenylguanidine (DPG) is one of a family of secondary accelerators manufactured at Flexsys Rubber Chemicals. The compound is produced in a number of physical forms *i.e.*, micronised powder, a dust-suppressed powder, and as 2 mm granules. Chemically it has the structure shown.



diphenylguanidine

There exists very little historical data on the biodegradability of DPG at the Flexsys manufacturing site, nor is there much information on the compound in the literature dealing with biological removal of DPG from wastewaters.

Tomlinson *et al.* (1965) studied the effects of various inhibitors on nitrification in the activated sludge process. A large number of compounds were reviewed amongst which were diphenylguanidine, aniline and guanidine. It was reported in the study that the concentration of guanidine and diphenylguanidine required to produce a 75% inhibition were 1.0×10^{-4} and 2.5×10^{-4} M, respectively, whereas aniline concentration required to produce the same 75% inhibition was 8.31×10^{-5} M. It is important therefore that the levels of aniline in the feed to the biological reactors be limited to prevent toxic shock and other effects to the biomass.

N-Substituted aromatics, such as nitroaromatics, azo dyes and aromatic amines, are important priority pollutants entering the environment primarily through anthropogenic activities associated with the industrial production of dyes, explosives, pesticides and pharmaceuticals. Aromatic amines may pollute surface waters via wastewater inputs and groundwater transport of the products of the metabolism of some pesticides and explosives (Rippen, 1990). Many aromatic amines have a high toxicological, carcinogenic, mutagenic and ecotoxicological potential (Fishbein, 1984; Sax *et al.*, 1984; Razo-Flores *et al.* 1997). It is important therefore that the biomass is capable of metabolising aromatic amines at these concentrations before further discharges into the river course take place.

5.2.1 Methodology

The DPG was spiked at 50 mg/l into the synthetic feed matrix and fed onto the biomass of reactor R3 over a ten-day period. Samples were collected at 24 hour intervals and each subjected to HPLC analysis using method HPLC 6 (see Chapter 2 for full analytical details). Solid phase extraction (SPE) was used to pre-concentrate each sample prior to HPLC analysis. The samples were passed over an Isolute ENV+ cartridge and isolated analytes were then eluted from the cartridge using acetonitrile (far UV grade). The extracts were analysed for residual DPG and associated metabolites.

5.2.2 Results of DPG degradation study

Initial samples were found on analysis to contain very little identifiable organic residues. Analysis of the effluent collected at the end of day three indicated that DPG was by then present at trace levels along with various unidentifiable polar metabolites. The concentration of DPG in the effluent collected at the end of day four was just over double that determined for the day three sample (see Figures 5.11 and 5.12 respectively).

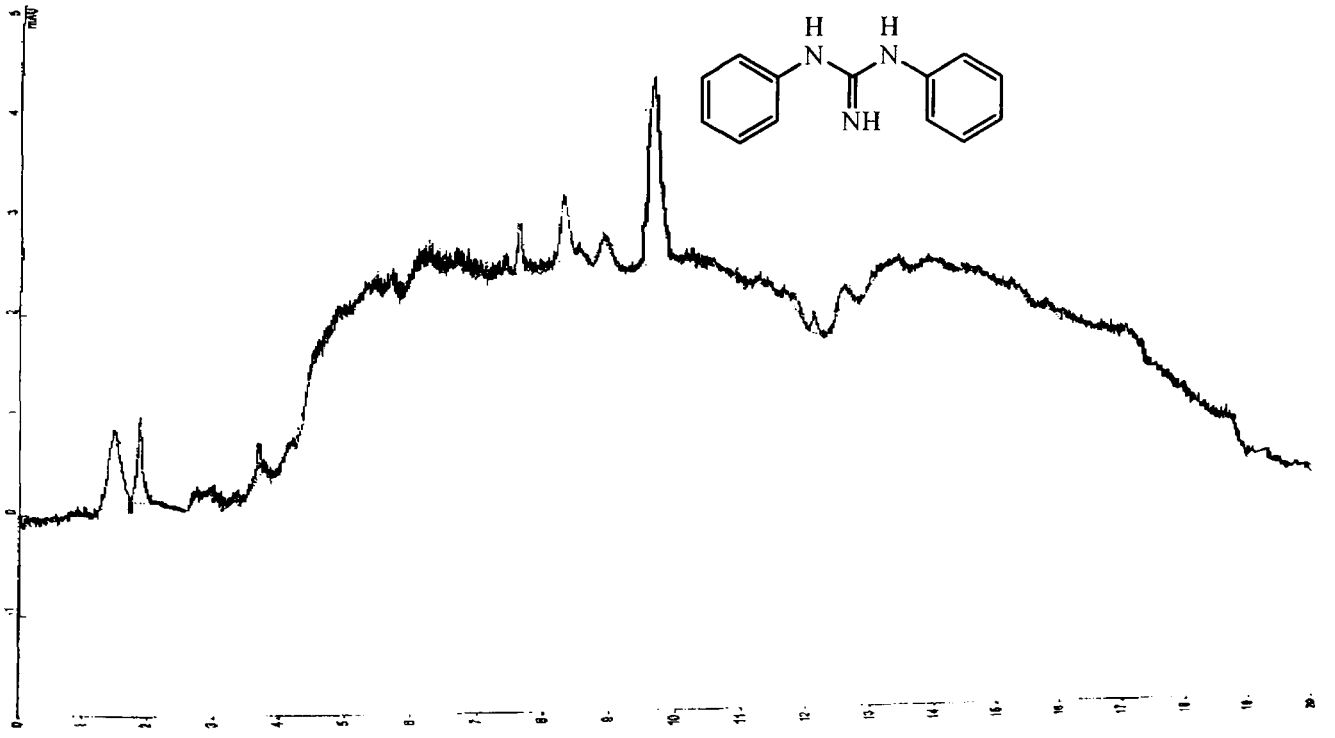


Figure 5.11 HPLC DPG 1. Day three of DPG degradation study.

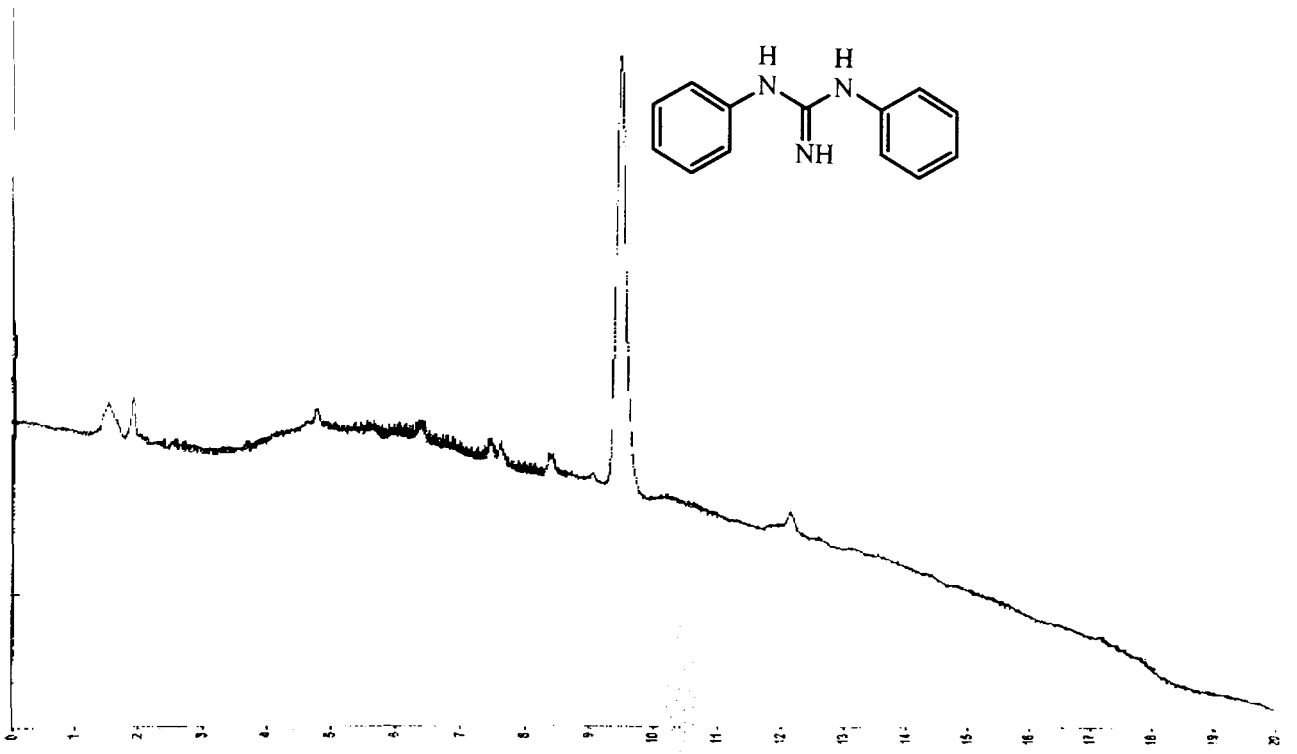


Figure 5.12 HPLC DPG 2. Day four of DPG degradation study.

During the course of the experiment the levels of DPG continued to increase in the effluent from reactor R3. Further metabolites were identified from day five onwards (Figure 5.13). Traces of aniline were detected in the effluent for the end of day five and continued to increase towards the end of the ten-day period. Diphenylurea (DPU), was detected at day six followed by diphenyl oxide (DPO) at day seven. By the end of day eight phenylurea (PU) was detected in the effluent.

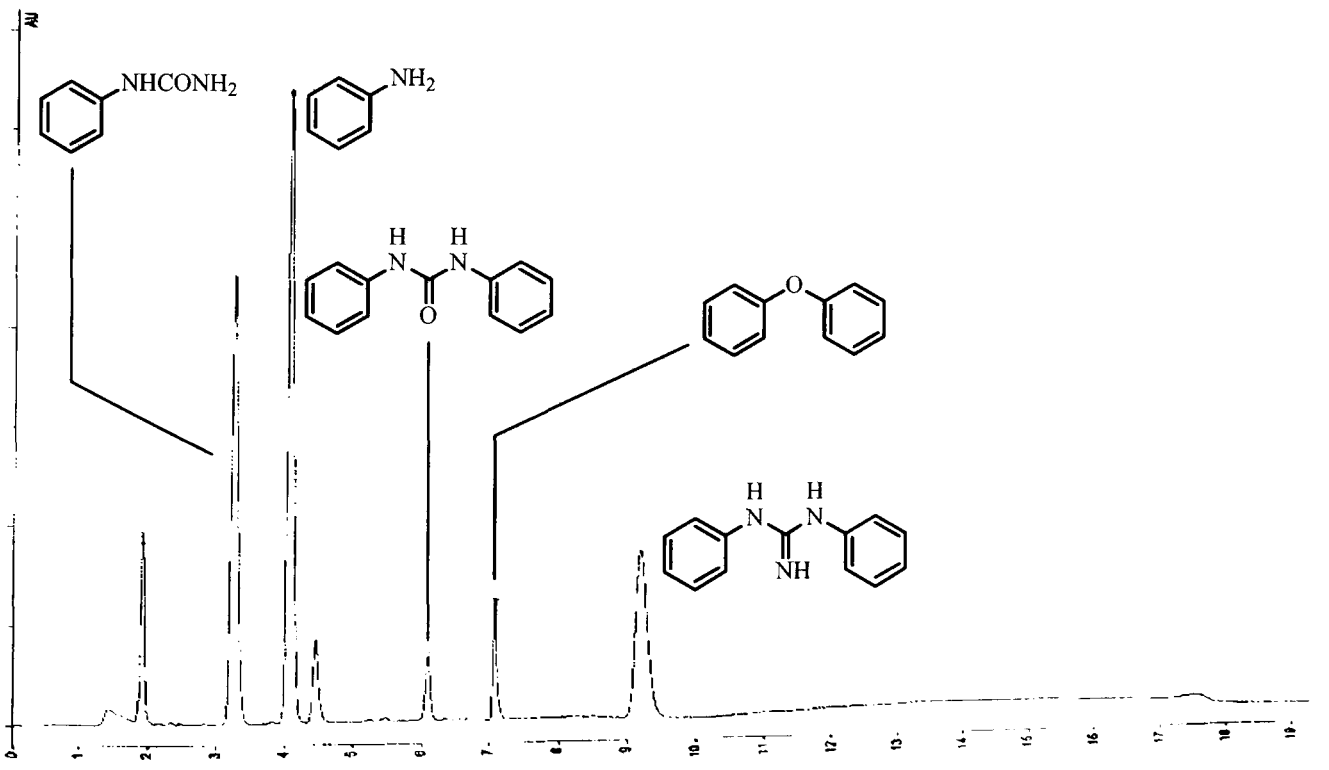


Figure 5.13 HPLC DPG 3. Analysis of the final day's effluent sample from reactor R3.

All HPLC peak area response values are presented in Table 5.2 and used to construct the relevant charts showing the various trends observed during the experiment (Figure 5.14).

Table 5.2 HPLC DPG analysis peak area response.

Day	Phenylurea	Aniline	Diphenylurea	Diphenyl Oxide	Diphenylguanidine
1	N.D	N.D	N.D	N.D	N.D
2	N.D	N.D	N.D	N.D	N.D
3	N.D	N.D	N.D	N.D	2948
4	N.D	N.D	N.D	N.D	6105
5	N.D	2515	N.D	N.D	15110
6	N.D	45682	36152	N.D	8279
7	N.D	86904	68605	9998	11362
8	10994	126424	101378	14902	16394
9	11641	139766	102439	17305	12115
10	11026	127293	96116	15173	10202

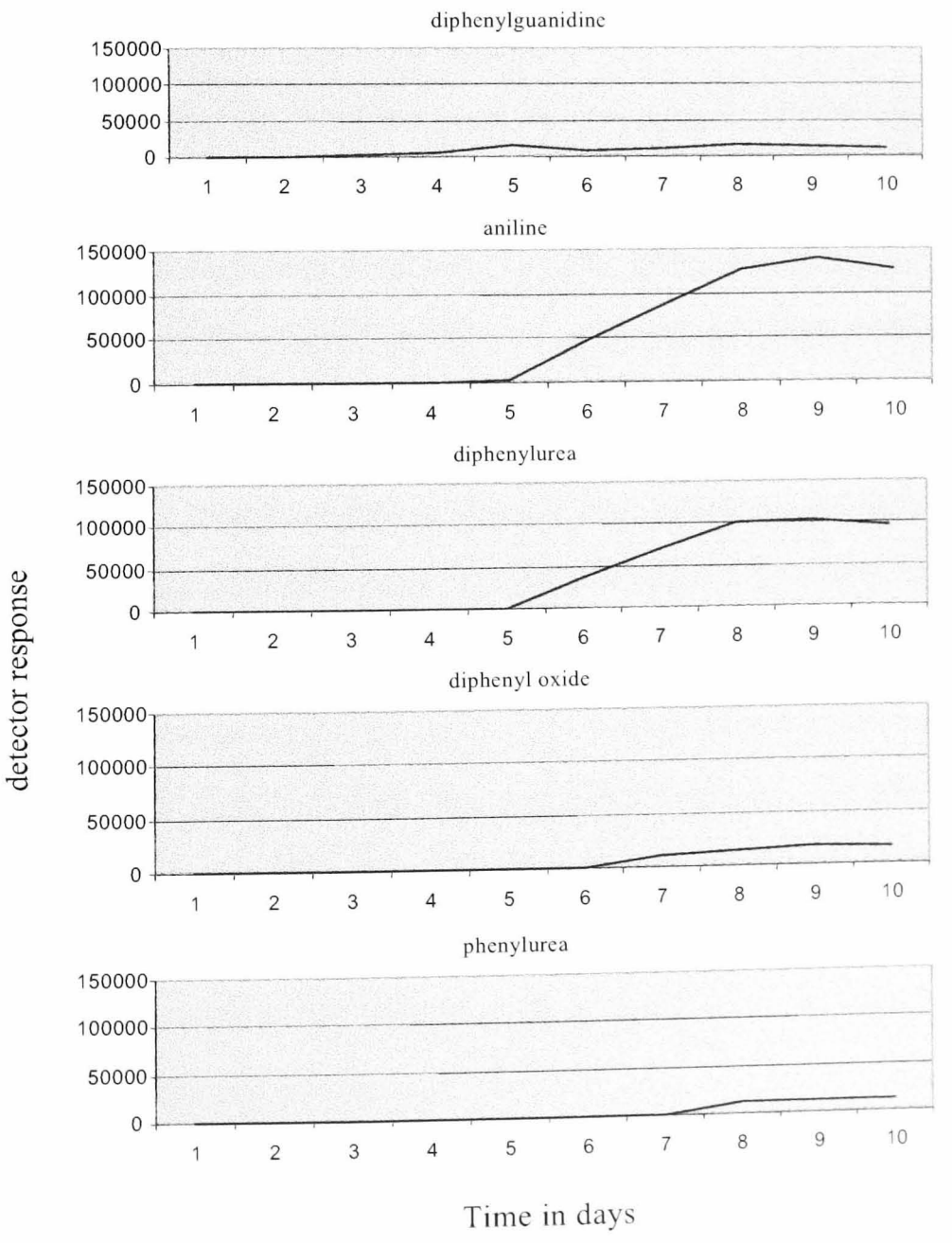
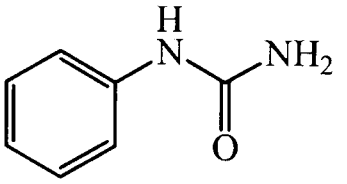
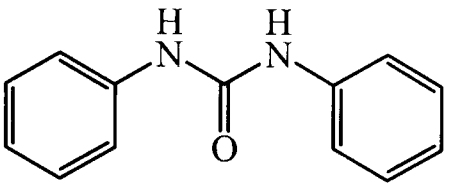


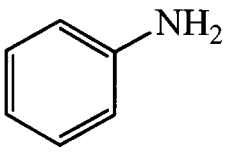
Figure 5.14 DPG HPLC analysis charts.



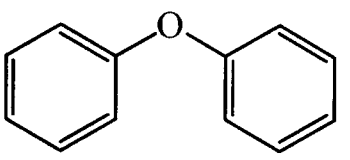
phenylurea



diphenylurea



aniline



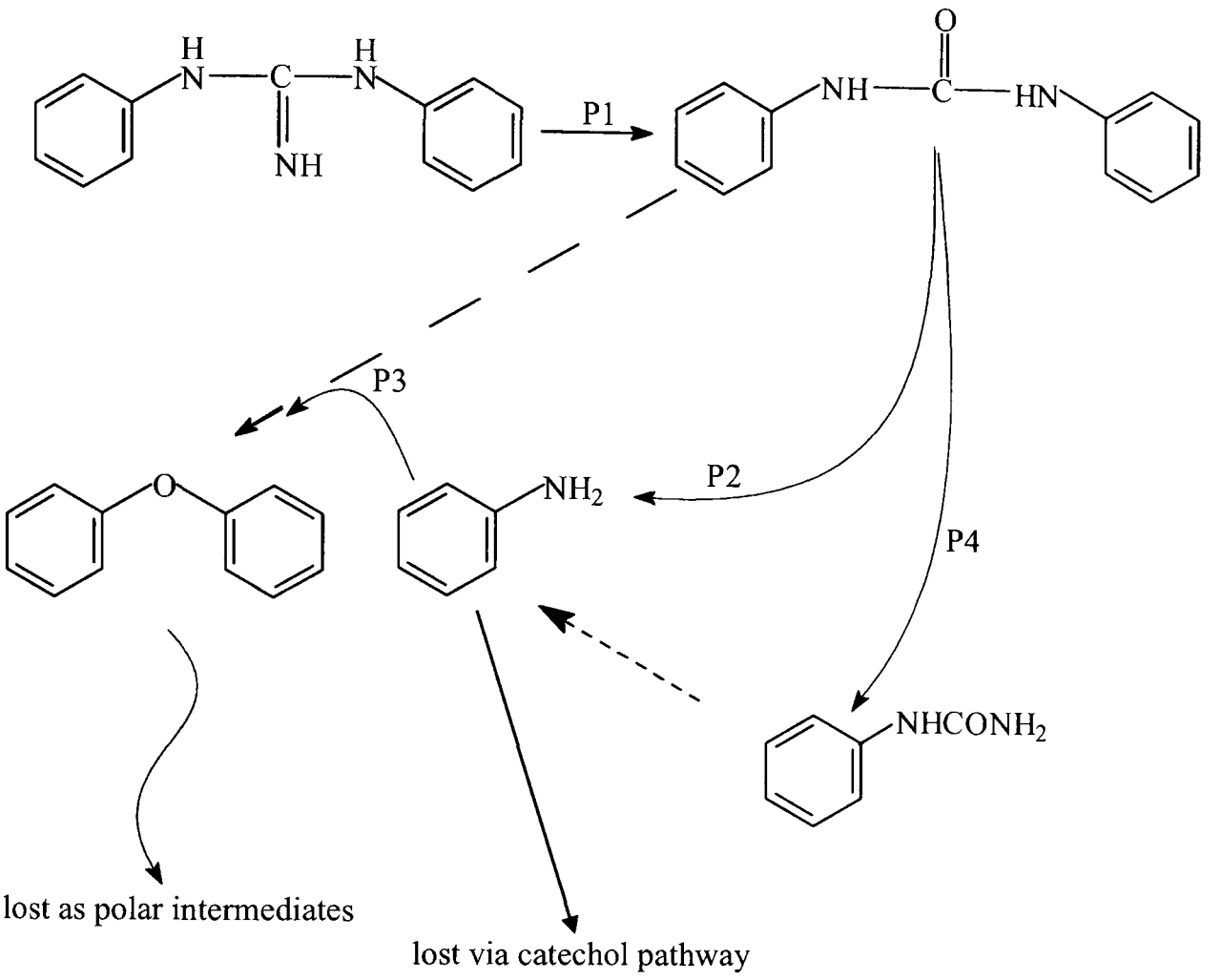
diphenyl oxide

5.2.3 Discussion of DPG degradation study

The lack of previous reports of studies into the degradation of DPG by aerobic bacteria makes it difficult to compare and contrast the findings of the present work. However one report specifically cited the effects of DPG on the nitrification process (see Tomlinson *et al.*, 1966). It was hitherto thought that DPG removal was rapid and complete; however, the above findings show that this is not the case.

The main wastewater treatment plant at Flexsys Ruabon site has a long history of treating wastewater containing relatively high concentrations of DPG, *ca.* 100–235 mg/l. Analysis of the discharge from the wastewater treatment plant would suggest that DPG at these concentrations is removed very efficiently, since typical detected concentrations for DPG are below 0.001 µg/l. However, following a prolonged absence of DPG from the feed to the biomass, as in the case of reactor R3, the necessary enzymes for DPG metabolisation are lost from the biomass. Following the reintroduction of DPG to the biomass significant levels of DPG are detected in the effluent confirming the above statement that the enzymes necessary for DPG removal are lost over time. However, once the required enzymes have been stimulated, the DPG is quickly metabolised as indicated by the production of a series of metabolites.

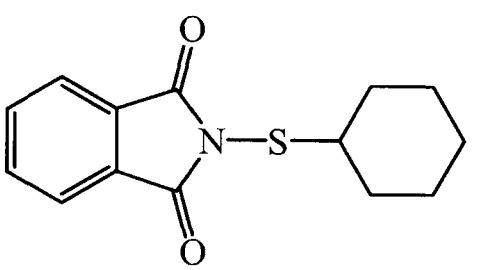
The following is proposed as a hypothetical pathway for the removal of DPG based on the above analytical data.



In the above scheme the pathway P1 gives rise to aniline which is observed in the effluent before any diphenylurea (DPU) is detected. The initial conversion rate of DPU to aniline is faster than the removal rate for aniline. DPU generation lags behind aniline generation hence no DPU is observed for 24 hours following onset of aniline production. The initial degradation pathway for DPU is via formation of aniline. Once this pathway is established, a secondary pathway to phenylurea (PU) is initiated. The PU generated thus is then further metabolised to produce aniline, hence the steady build up of aniline within the system. Once the requisite enzymes for aniline removal have been established, the rate of aniline metabolisation exceeds the combined conversion rates of DPU and PU to aniline. The net effect is the slow removal of aniline from the system shown by the downward trend in the aniline graph.

5.3 Biodegradation of cyclohexylthiophthalimide

The compound cyclohexylthiophthalimide, commonly referred to as PVI, is a prevulcanisation inhibitor manufactured by only a few rubber chemical manufacturers. Of these manufacturers only Flexsys Ruabon UK treats wastewater from the PVI process using the activated aerobic sludge process. As a consequence there is little literature dealing with the direct study of its aerobic metabolisation and of the associated metabolites. This work therefore is unique both in its approach and in the results obtained for PVI biodegradation by a mixed consortia of aerobic bacteria.



cyclohexylthiophthalimide (PVI)

In terms of biological degradation very little is known about PVI. Despite exhaustive literature searches no published data could be found concerning industrial aerobic degradation of PVI using the activated sludge process. The situation was no different regarding the various metabolites generated during the study. The results of this study have therefore generated a unique data set relating to the specific metabolisation of PVI and associated metabolites by an aerobic biomass.

The experiment was run using the biomass of reactor R3 following a wash out from previous studies and re-acclimation to the base synthetic feed matrix. As in previous experiments the substrate to be studied was spiked into the synthetic feed at 50 mg/l and fed onto the biomass of reactor R3 over a ten-day period. Samples were taken at the end of each 24 hour period and analysed by GCMS for residual PVI and derived metabolites.

5.3.1 Results of PVI degradation study

Analysis of the first 24 hour effluent sample by GCMS clearly showed the PVI molecule to be very rapidly transformed to a series of simpler species (Figure 5.15). The main compound present was found on analysis to be phthalimide, which was amenable to further biological breakdown. The phthalimide was no longer detectable in the effluent from day seven onwards.

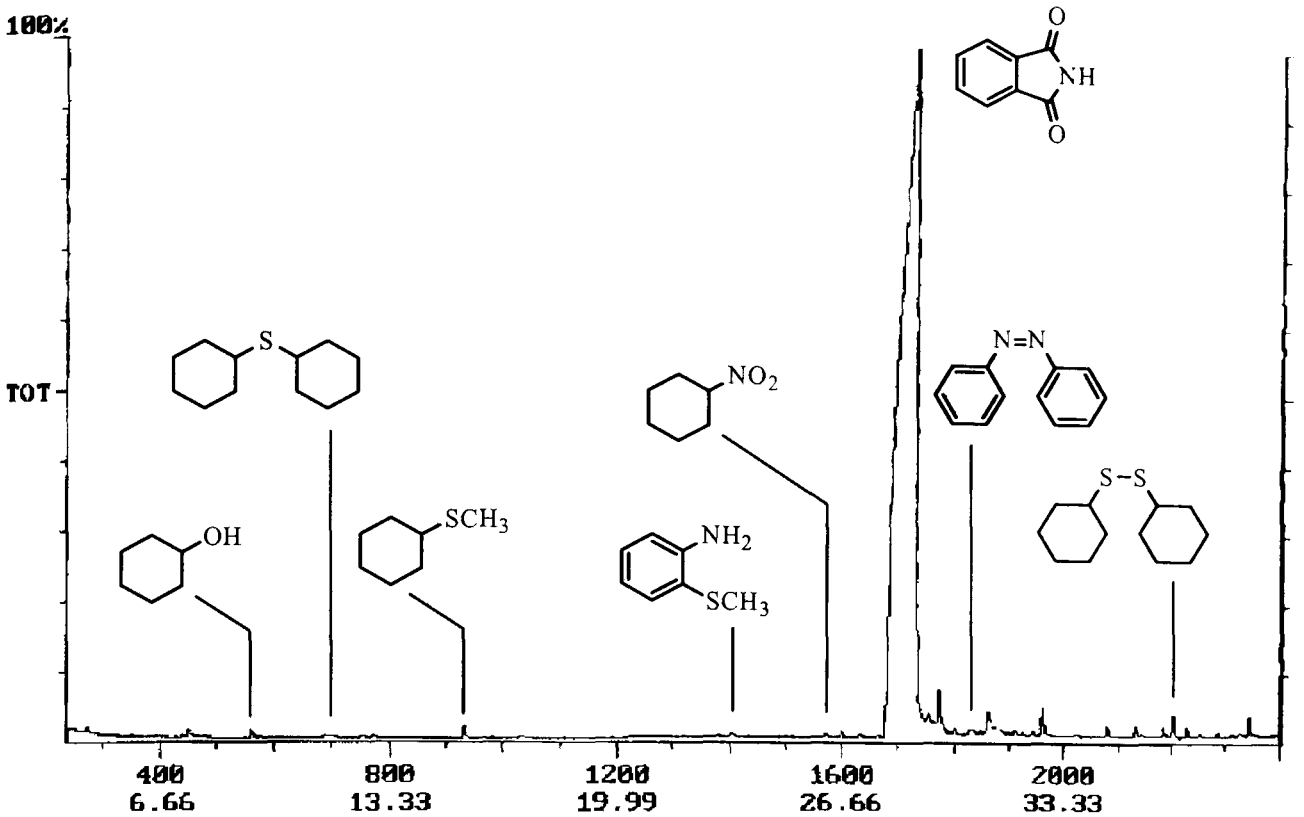


Figure 5.15 GCMS PVI 1. The first 24 hours.

Other species generated by the biomass included dicyclohexyl sulphide and the corresponding disulphide, methylcyclohexane, nitrocyclohexane and cyclohexanol. Two aromatic species were also formed, namely 2-(methylmercapto)aniline and azobenzene.

Figure 5.16 shows how rapid the biotransformation of phthalimide and the onset of dicyclohexyl sulphide generation was under the conditions of the experiment.

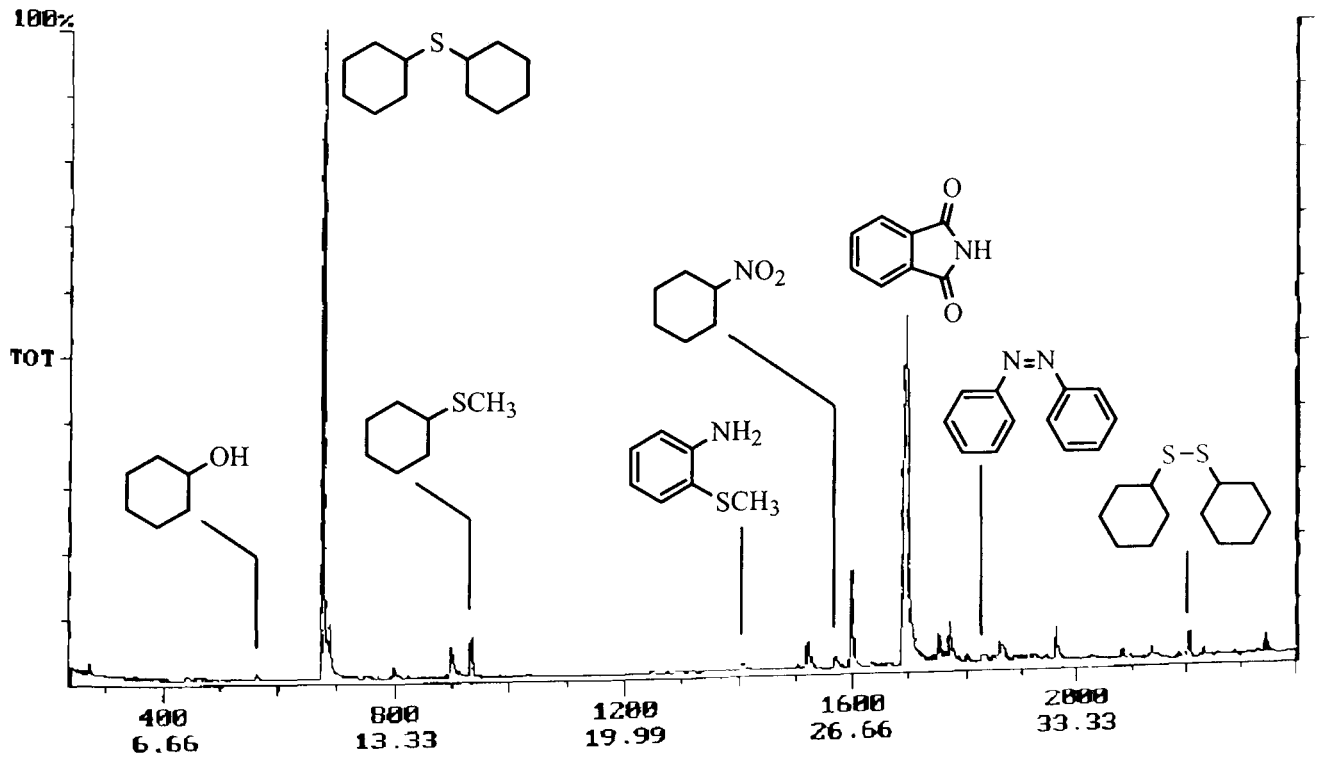


Figure 5.16 48 hours of PVI metabolisation.

The GCMS analysis results are given in Table 5.3 and presented graphically in Figure 5.17. This latter clearly shows the relationship between the onset of 2-(methylmercapto)aniline production and the rapid conversion/removal of nitrocyclohexane.

Table 5.3 PVI metabolisation study GCMS peak area response data.

Day	1	2	3	4	5	6	7	8	9	10
Phthalimide	64257320	2582291	693205	1023	622	0.5	0.5	0.5	0.5	0.5
DMK	1036560	116856	80358	84280	8558266	68841	66117	67982	65293	64781
Dicyclohexyl sulphide	12113	2207255	1913192	2613757	4406060	2577930	1394385	867455	53296	44817
Dicyclohexyl disulphide	65436	69669	44403	44306	50086	35265	28282	22041	14119	10270
Methylthiocyclohexane	51205	103544	94545	56791	22677	20478	19623	17992	16036	14856
Cyclohexanol	43155	20700	6875	958	0.5	0.5	0.5	0.5	0.5	0.5
2-Methylmercaptoaniline	21063	37463	40468	18656	34586	137368	152085	149786	141055	138742
Nitrocyclohexane	14256	61647	70480	81821	84707	13915	11251	8043	5115	1948

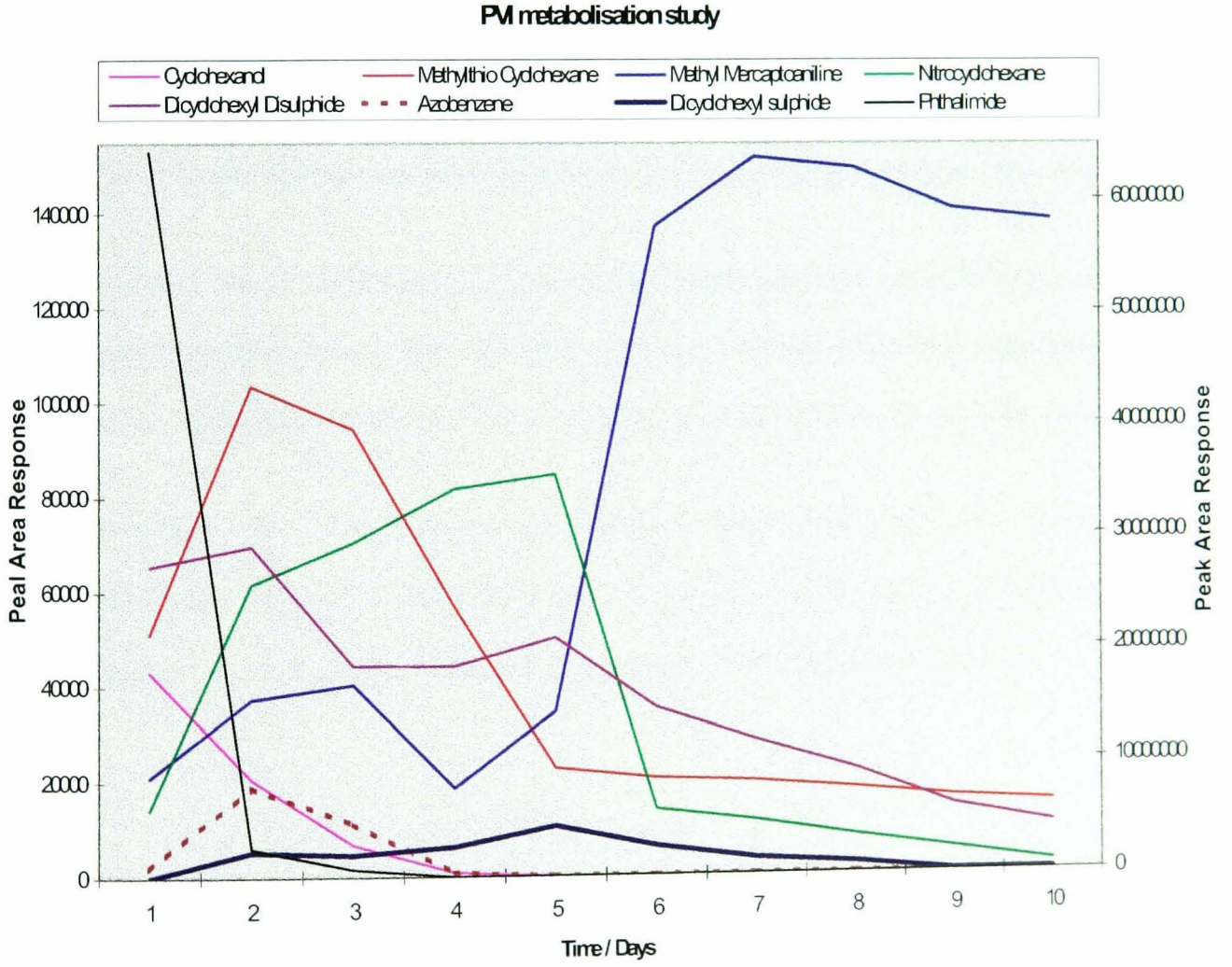
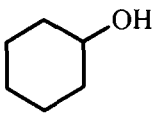
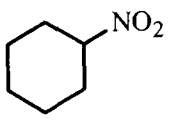


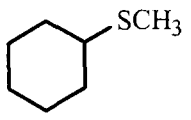
Figure 5.17 GCMS analysis chart from peak area analysis data given in Table 5.3.



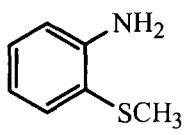
cyclohexanol



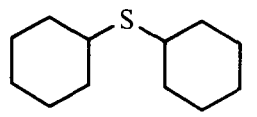
nitrocyclohexane



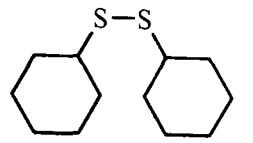
methylthiocyclohexane



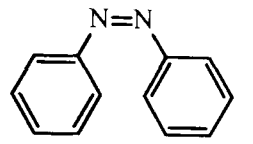
2-(methylmercapto)aniline



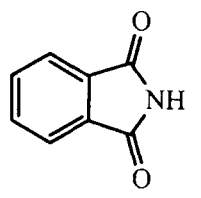
dicyclohexyl sulphide



dicyclohexyl disulphide



azobenzene



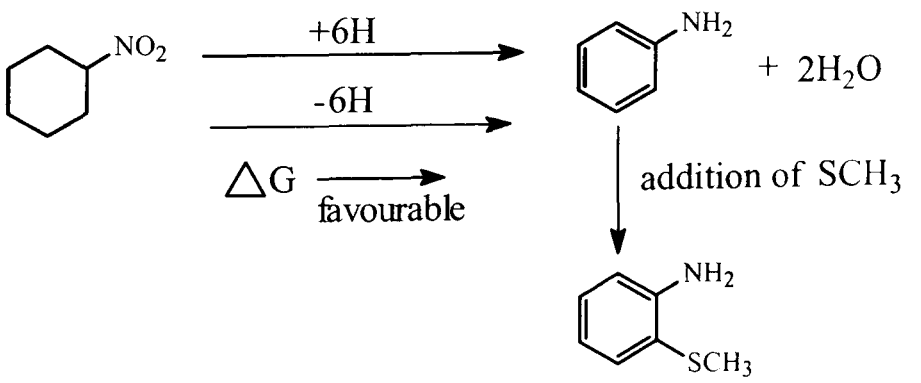
phthalimide

5.3.2 Discussion of PVI degradation study

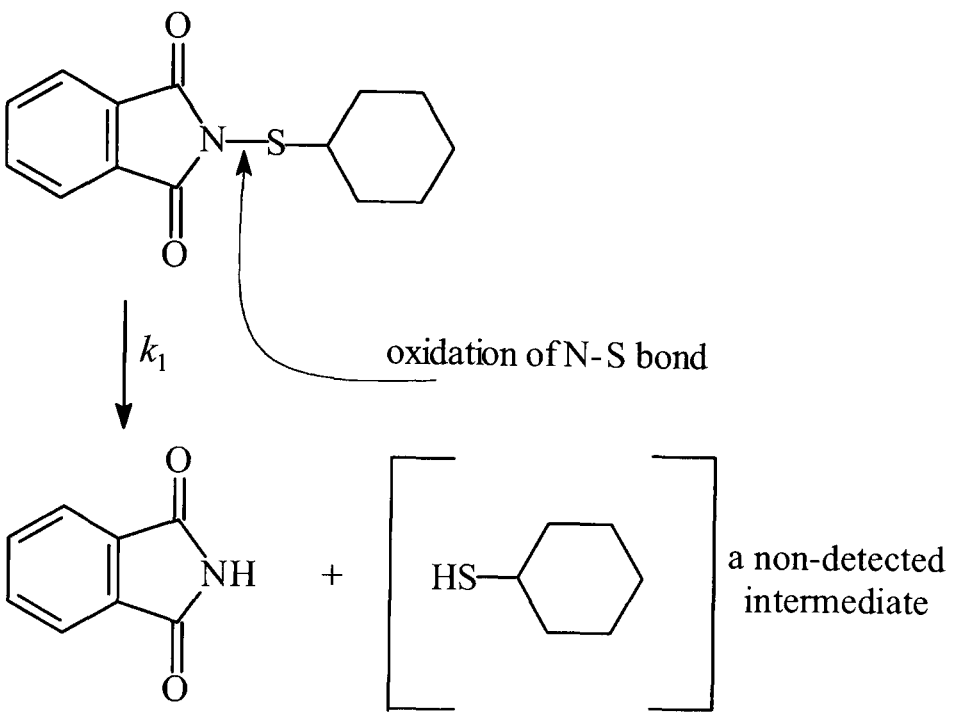
What is clearly evident from the study is that the compound PVI is very easily and rapidly broken down under the aerobic conditions prevailing inside bioreactor R3. Analysis of the main water treatment plant effluent also supports this finding, as no PVI and very little if any phthalimide are found in the effluent from the plant even when there are significant levels of PVI in the feed to the biological reactors (significant being in excess of 50 mg/l).

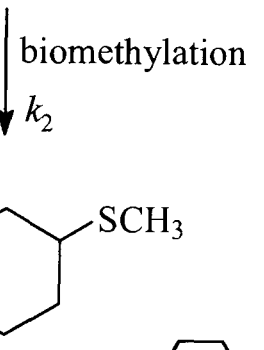
Trace amounts of cyclohexanol, 2-(methylmercapto)aniline, dicyclohexyl sulphide and dicyclohexyl disulphide are routinely found in the effluent from the main water treatment plant, further supporting evidence for the findings of the laboratory reactor study.

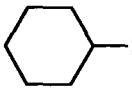
The generation of 2-(methylmercapto)aniline from PVI has not been previously investigated nor reported. The following is a proposed mechanism for its generation following formation of nitrocyclohexane along the PVI metabolic pathway.



PVI breakdown is very rapid and complete with little residual PVI evident after 48 hours. Furthermore, its removal parallels the formation of phthalimide. A possible mechanism for this hypothesis is as follows. A rapid oxidation of the N–S bond occurs to generate phthalimide together with cyclohexyl mercaptan (a non-detected intermediate along the pathway to methylthiocyclohexane) which is then followed by biomethylation to generate methylthiocyclohexane.



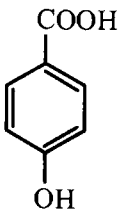


In the above scheme k_2 >>> k_1 hence the species  is not observed

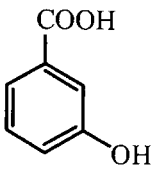
It is both fortunate and unfortunate that there appears to be no previously reported evidence to support or disprove the above hypothesis. Analytical data collected for the laboratory biological reactor is however supported by similar findings from analysis of effluent from the main wastewater treatment plant when being fed with a matrix containing high levels of PVI, *ca* 50–100 mg/l. A similar set of metabolites was observed on analysis.

5.4 Biodegradation of *para*-hydroxybenzoic acid

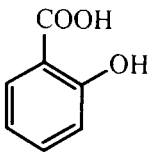
p-Hydroxybenzoic acid (PHBA) is a member of the phenolic acid group, of which there exists three isomers. The commercially most important member of the group is the *ortho*-isomer, commonly known as salicylic acid (Finar, 1973). However, the *para*- isomer finds use as a biocide in cosmetic formulations and in certain soft food preparations. PHBA is used both as the free acid and as the methyl and/or ethyl esters, commonly known as methyl and ethyl parabens respectively (Figure 5.18).



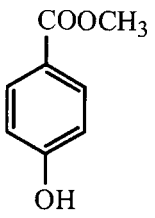
p-hydroxybenzoic acid

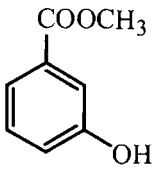


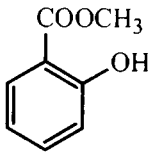
m-hydroxybenzoic acid



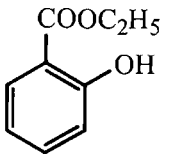
o-hydroxybenzoic acid

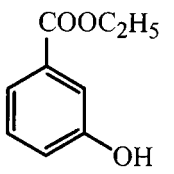


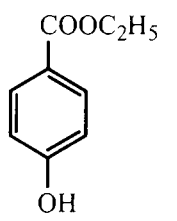




methyl esters







ethyl esters

Figure 5.18 Phenolic acids and their methyl and ethyl asters. The esters of the *para* isomer are also called methyl parabens and ethyl parabens respectively (see Section 5.5).

The product PHBA has a long history of manufacture at the Flexsys Rubber Chemicals site in North Wales. However, as it has traditionally been thought to be readily biodegradable, no previous studies have been carried out into its metabolisation. Analysis of the effluent discharges from the PHBA manufacturing plant revealed that concentrations of PHBA exceeded 3000 mg/l. However, analysis of effluent from the wastewater treatment plant showed PHBA concentrations below the current limit of detection (0.1 µg/l) confirming that it is indeed readily biodegradable.

In order to determine how quickly the removal of PHBA occurs and by what mechanism, the following experiment was carried out. The biomass of reactor R3 was fed a synthetic effluent (the base matrix used in all previous studies), spiked with 100 mg/l of pure PHBA, over a ten-day period. Samples of effluent from the reactor were collected at the end of each 24 hour period and analysed by HPLC and GCMS for residual PHBA and generated metabolites.

5.4.1 Results of PHBA degradation study

HPLC analysis of the first sample using HPLC Method 8 revealed that PHBA was almost completely removed from the feed within the first 24 hours. The results indicated that biomethylation had occurred very quickly leaving only a trace of PHBA along with small amounts of *cis,cis*-muconic acid, phenol and ethyl hydroxybenzoate (Figure 5.19).

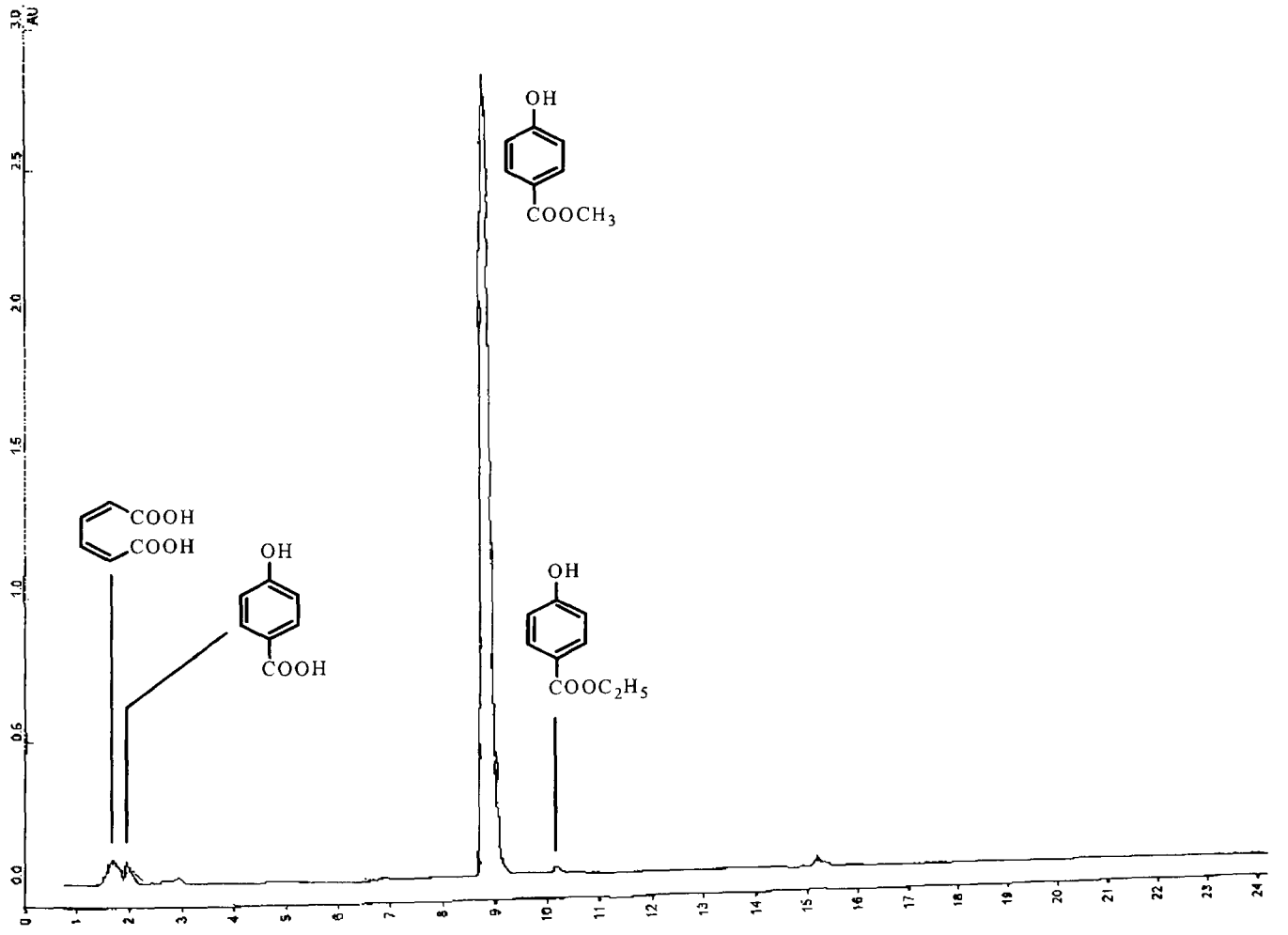
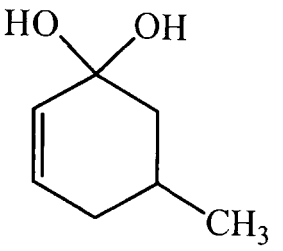


Figure 5.19 HPLC PHBA. The first 24 hours.

GCMS analysis confirmed the results of the initial HPLC analysis. Further metabolites were identified via mass spectral analysis of the various peaks in the chromatogram. Significant metabolites identified by GCMS were isovaleric acid, *p*-cresol, 2-

ethylhexanoic acid, benzoic acid, phthalic anhydride and a polar compound with the following structure.



This was the most significant metabolite identified by GCMS next to methyl parabens as can be seen from Figure 5.20.

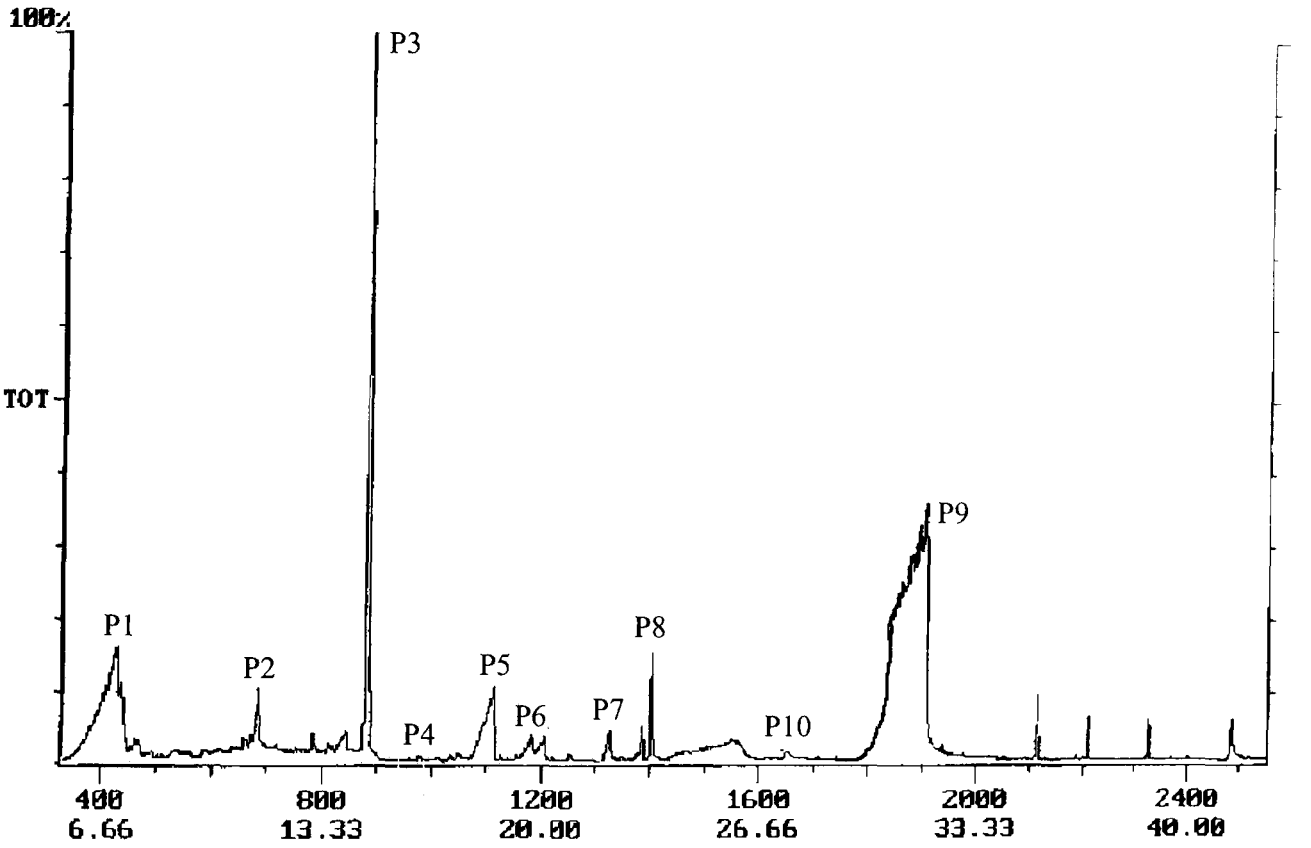


Figure 5.20 GCMS PHBA. The first 24 hours. Key: P1 isovaleric acid; P2 phenol; P3 unknown polar compound; P4 *p*-cresol; P5 2-ethylhexanoic acid; P6 benzoic acid; P7 Benzene acetic acid; P8 phthalic anhydride; P9 methyl parabens; P10 PHBA.

HPLC analysis of subsequent samples showed little change in the composition of generated metabolites. The concentration of PHBA remained low during the early part of the experiment. and the concentration of both methyl and ethyl parabens continued to increase during the course of the experiment. However, analysis of effluent collected from day six onwards indicated that the pathway initially operating in which PHBA was methylated was being inhibited and that a backward reaction mechanism had been initiated. This back reaction produced increasing amounts of PHBA and phenol (Figure

5.21). This is discussed in more detail in Section 5.5 in relation to the methyl parabens study.

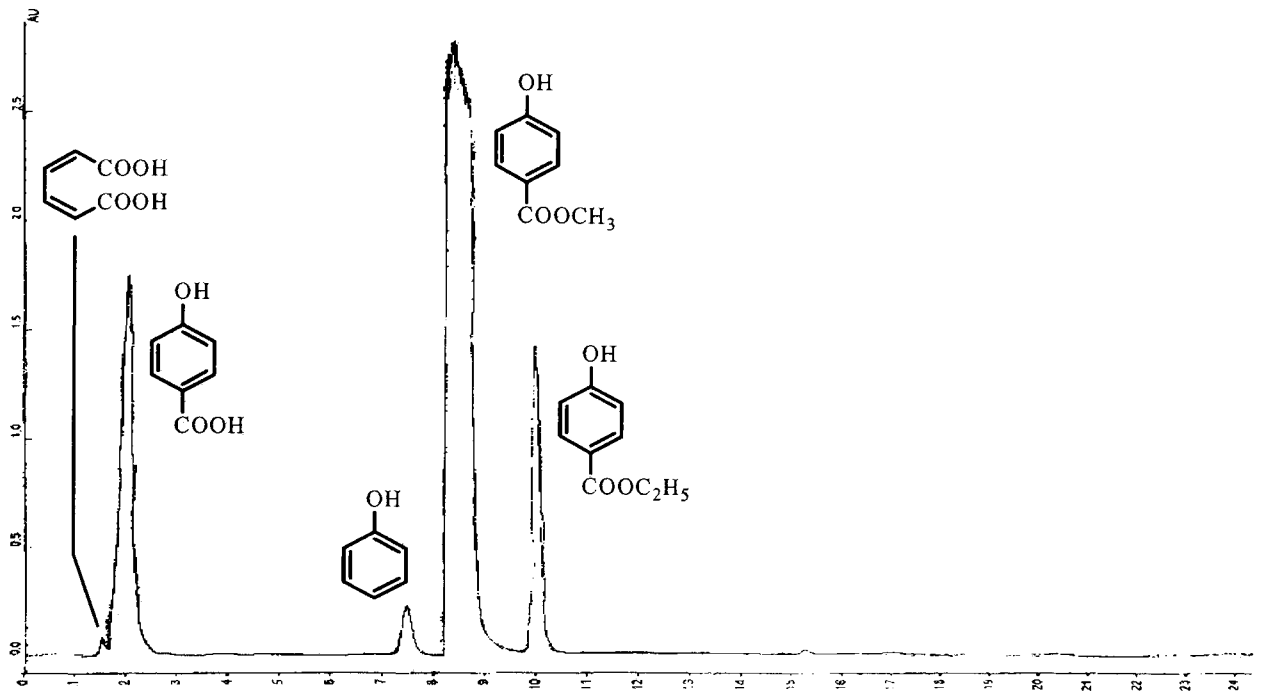


Figure 5.21 HPLC PHBA Day 6.

HPLC analysis of the day ten effluent sample confirmed that the levels of both PHBA and phenol had increased over the latter part of the experiment (Figure 5.22).

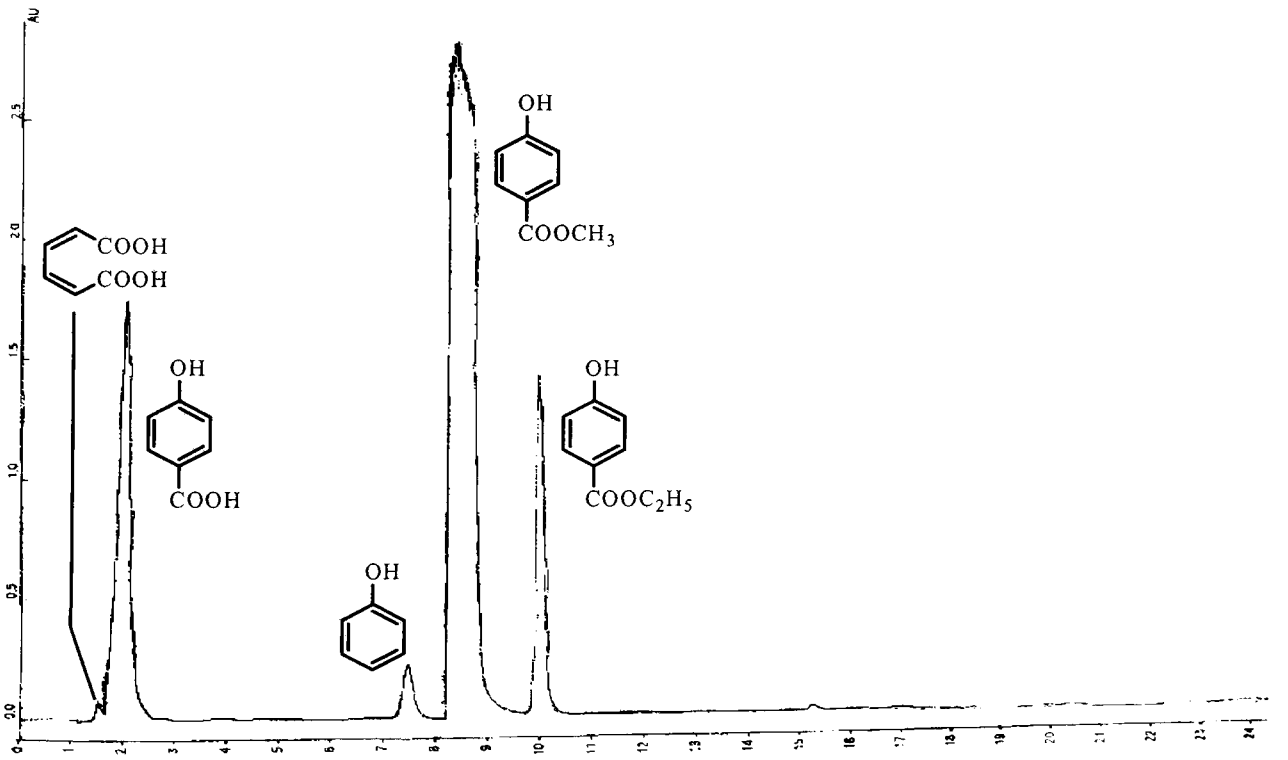


Figure 5.22 HPLC PHBA Day 10.

The point at which the back reaction appears to have been initiated can be seen from the chromatogram shown in Figure 5.23.

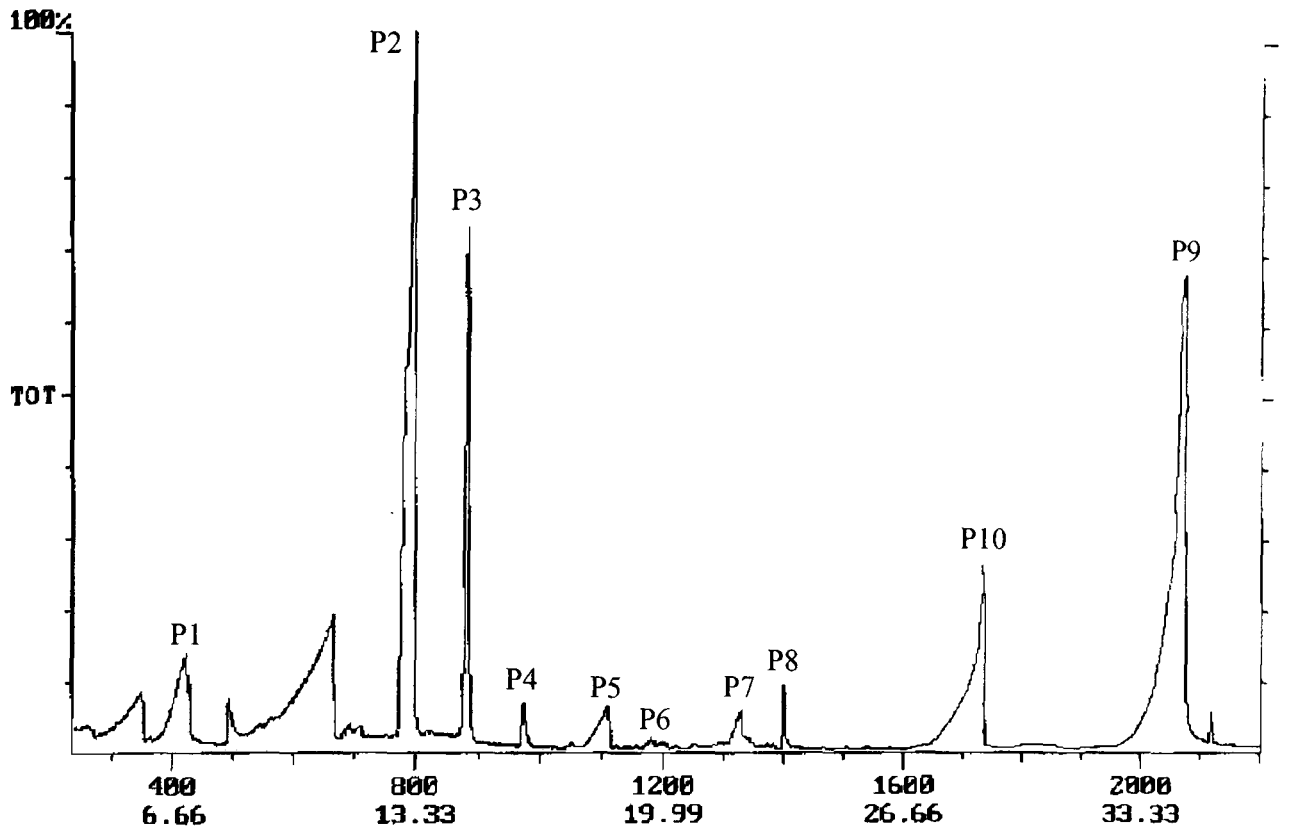


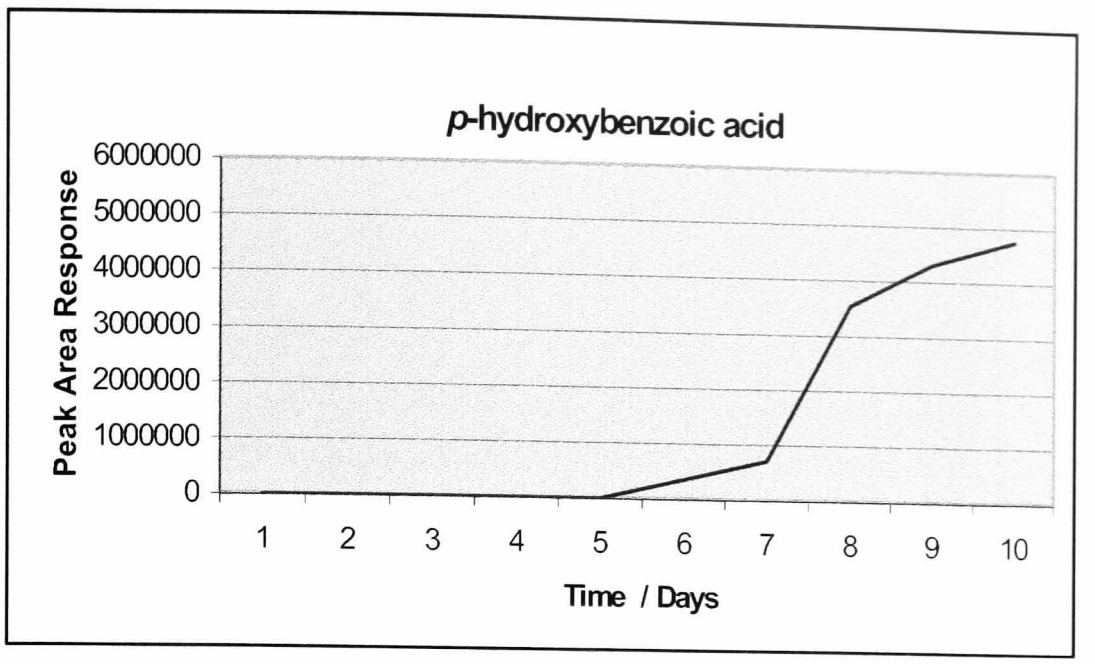
Figure 5.23 GCMS PHBA Day 5. Key: P1 isovaleric acid; P2 phenol; P3 unknown polar compound; P4 *p*-cresol; P5 2-ethylhexanoic acid; P6 benzoic acid; P7 benzene acetic acid; P8 phthalic anhydride; P9 methyl parabens; P10 PHBA.

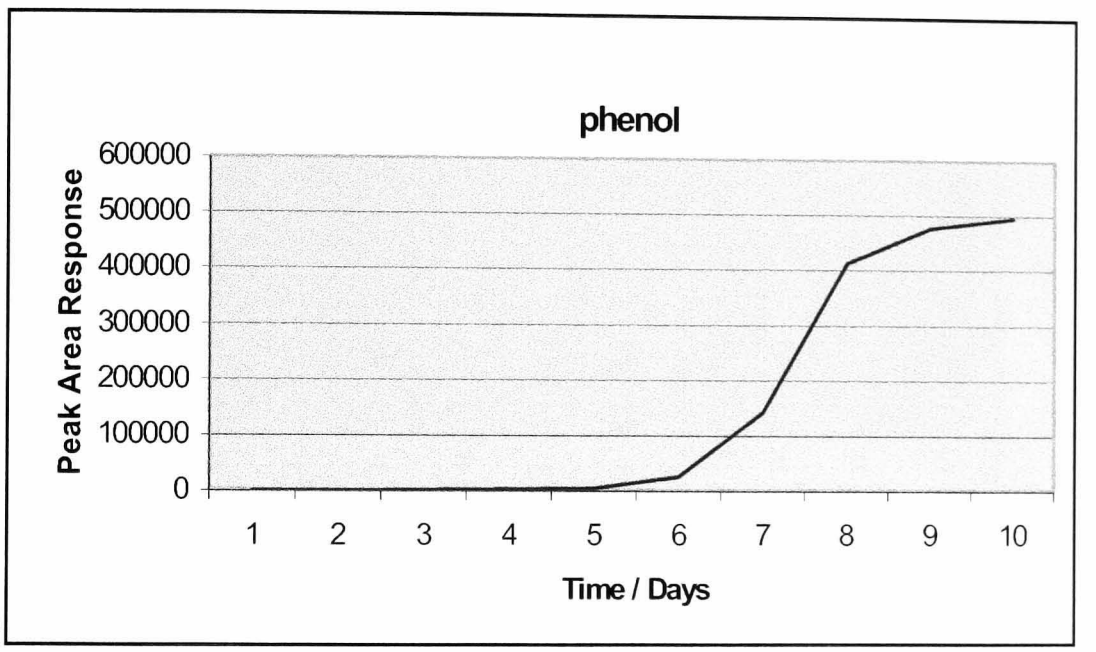
All HPLC analysis results are tabulated in table 5.4 below along with the associated charts in Figures 5.24 and 5.25. The data are presented in a series of aligned charts for ease of comparison through the course of the experiment, the three components; PHBA, phenol and methyl parabens are presented in figure 5.24 whereas the components *cis*,*cis*-muconic acid, catechol and ethyl parabens are drawn in figure 5.25 respectively.

Table 5.4 HPLC analysis data for PHBA study.

	Peak Area Response Values*					
Day	1	2	3	4	5	6
1	41063	3514	0.01	353	4465840	37705
2	41105	3281	0.01	496	8734493	46252
3	46239	4865	298	1161	9098413	98627
4	43118	4297	1366	2369	9645721	312647
5	45263	4611	6450	4153	10536402	984570
6	49572	352682	39119	25053	10609375	1036355
7	53662	714475	42939	142825	10159560	1867820
8	41076	3551286	0.01	413311	9126731	1930183
9	38643	4313627	0.01	477088	8484782	3029574
10	42557	4764528	1507	496163	6711625	3174118

*Key: 1 *cis*,*cis*-muconic acid; 2 *p*-hydroxybenzoic acid; 3 catechol; 4 phenol; 5 methyl parabens; 6 ethyl parabens.





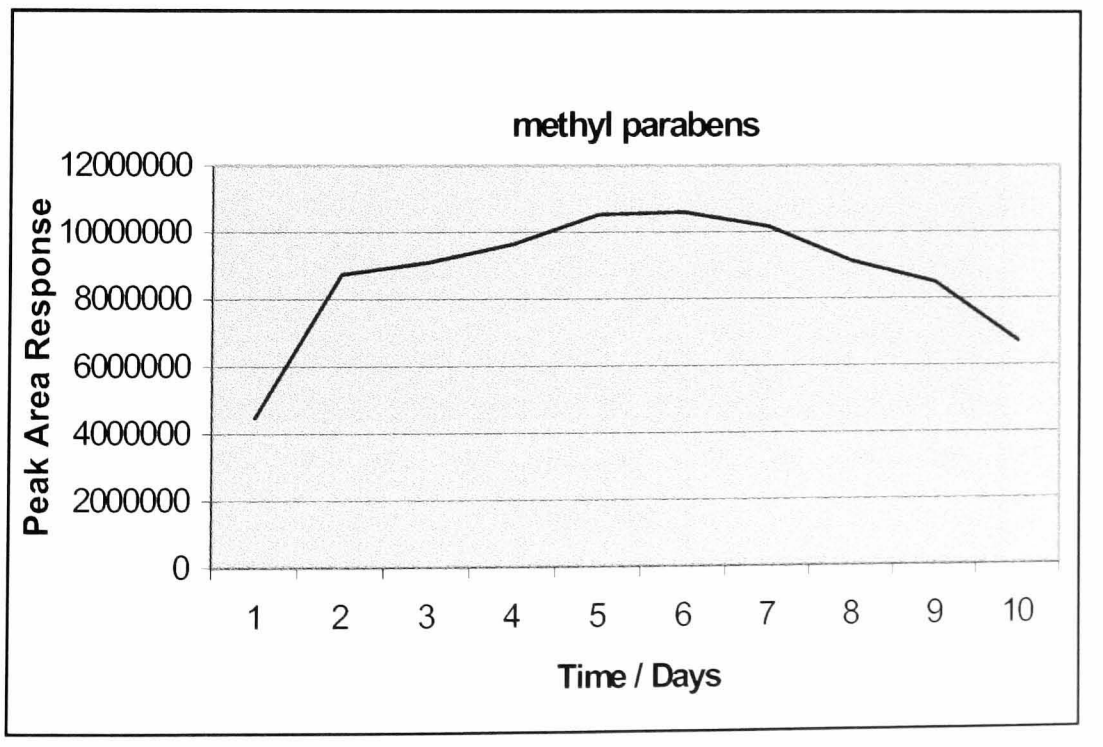
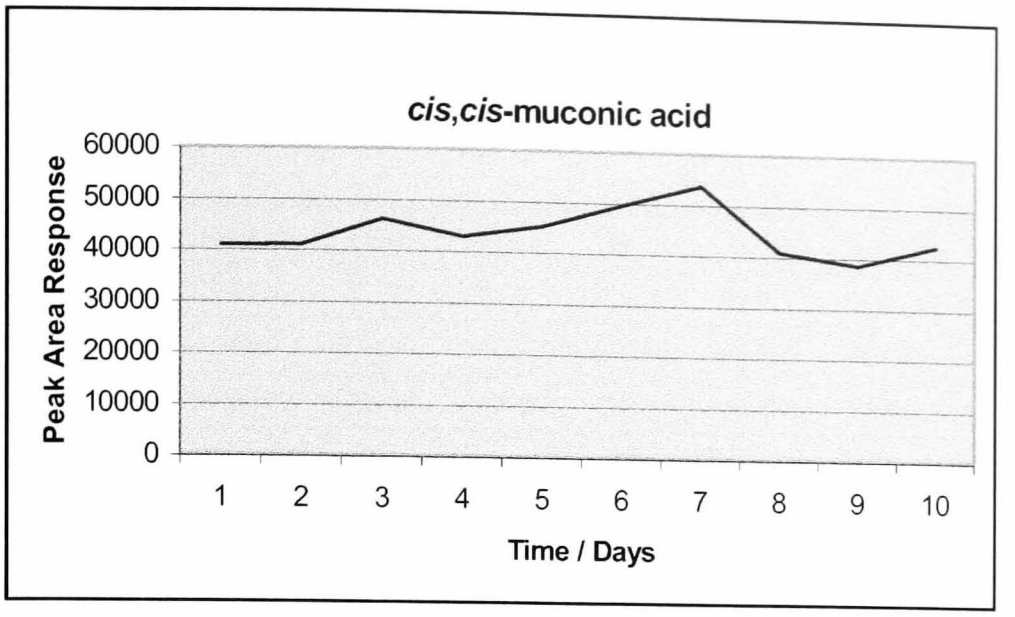
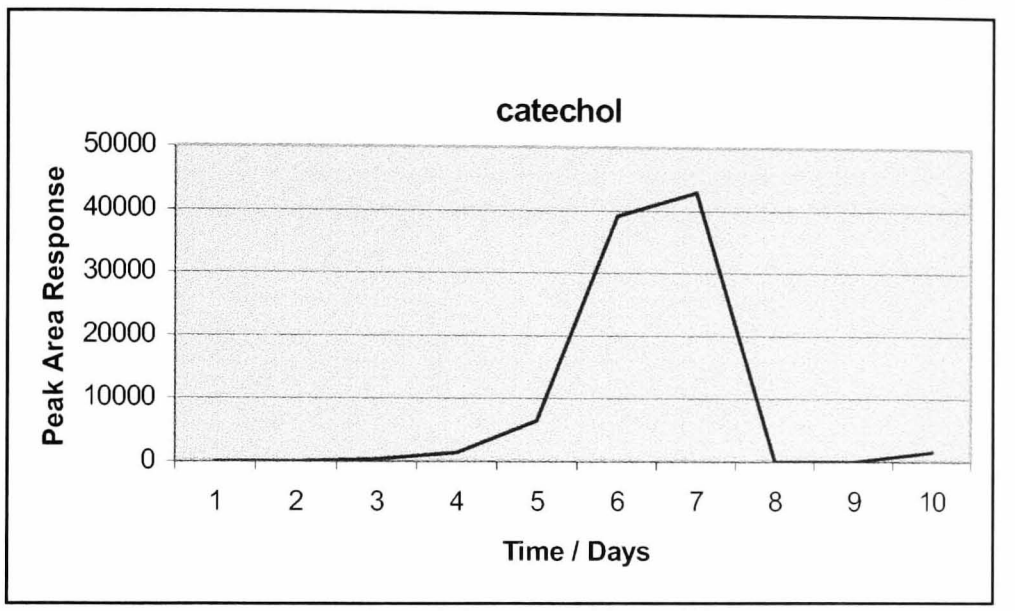


Figure 5.24 HPLC data charts for *p*-hydroxybenzoic acid, phenol and methyl parabens.





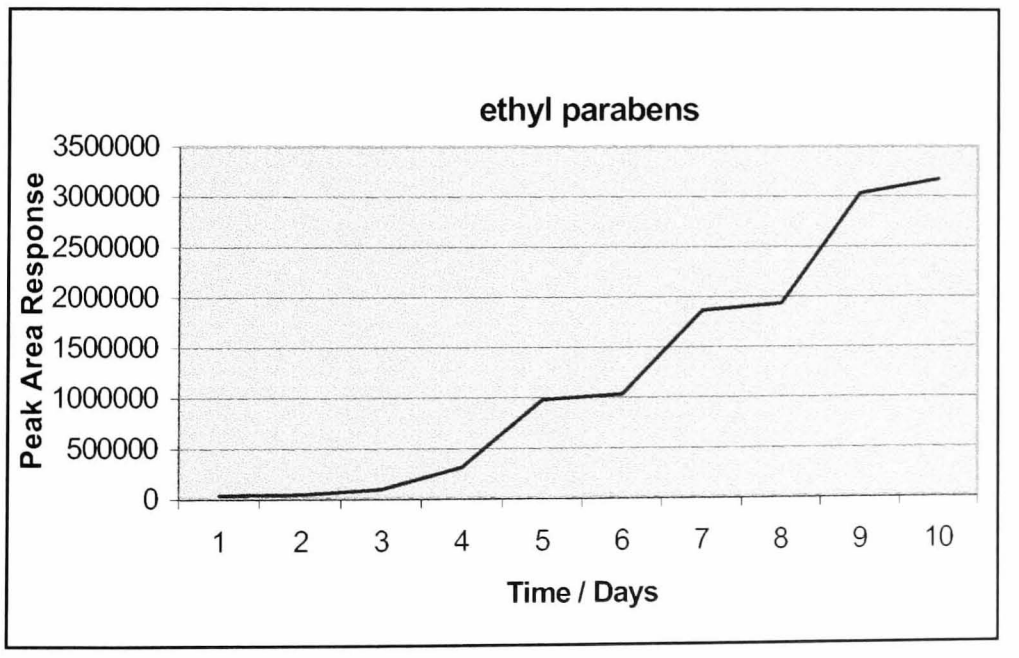


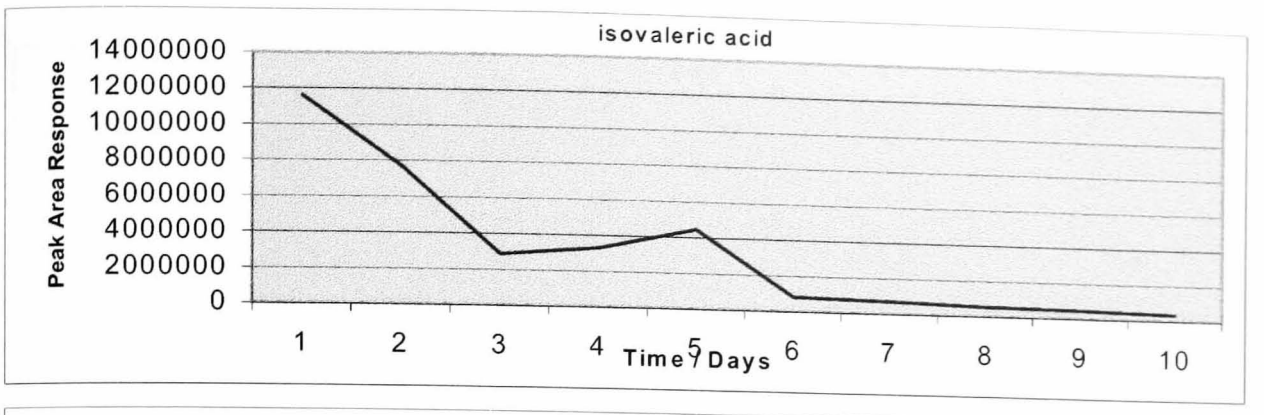
Figure 5.25 HPLC data for *cis*,*cis*-muconic acid, catechol and ethyl parabens.

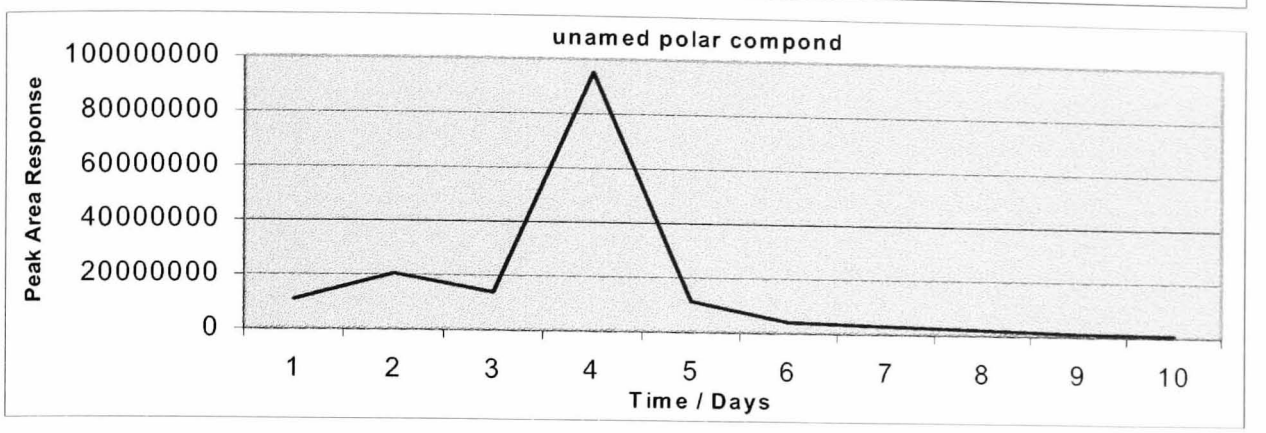
Table 5.5 contains peak area response values for identified metabolites by GCMS analysis of effluent from the PHBA metabolisation study and Figures 5.26 and 5.27 show the results in graphical form.

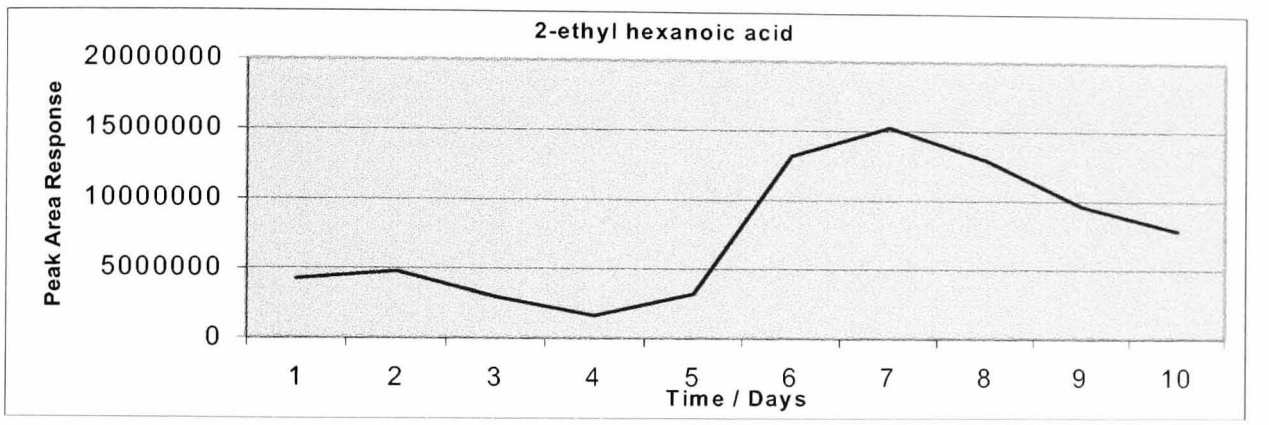
Table 5.5 GCMS analysis data from PHBA metabolisation study.

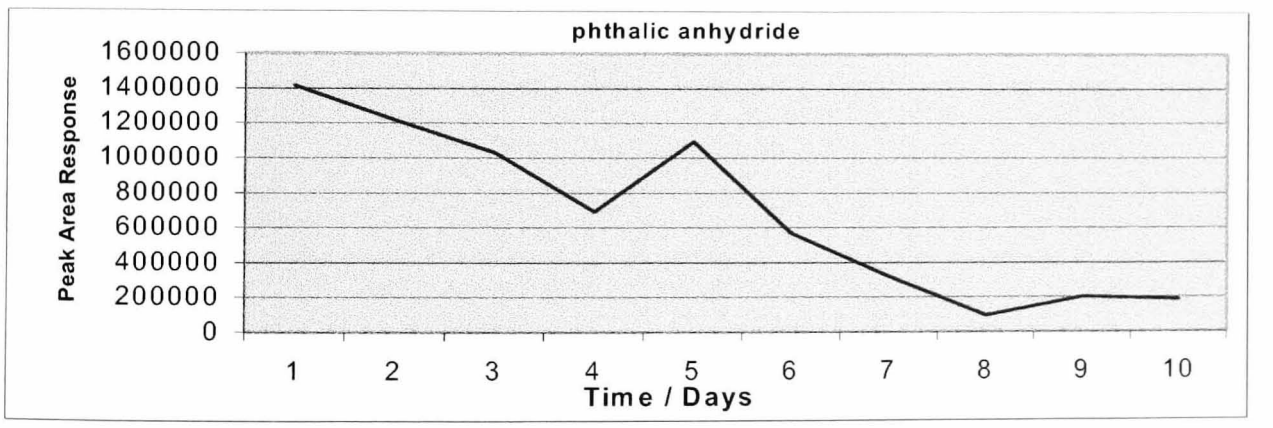
	Peak Area Response Values									
Day	1	2	3	4	5	6	7	8	9	10
1	11639925	270275	10878515	68844	4255525	139196	811793	1415326	54623410	153902
2	7738275	8230798	20561363	215229	4797964	193717	615896	1218703	125219437	164127
3	2866555	13727326	13856553	365897	2983324	250905	253234	1029494	149633705	169868
4	3289182	24370313	95146830	452516	1615673	259156	352703	689526	182715185	184765
5	44656755	39065950	11467683	1336591	3230436	183480	2024650	1093066	220546216	189863
6	823529	20758900	4082770	331286	13180500	58113	121819	567197	184458677	378416
7	714266	18765315	3014691	302561	15261302	41236	98711	319255	135215442	586672
8	551743	15215693	2147785	276411	12941733	32549	72543	96117	92285647	935738
9	462513	12008619	1116871	202537	9641552	10167	41762	201549	68336511	1416697
10	357842	864473	872459	187653	7866291	8846	20344	187651	49347755	1925635

*Key: 1 isovaleric acid; 2 phenol; 3 unknown polar compound with structure; 4 *p*-cresol; 5 2-ethylhexanoic acid; 6 benzoic acid; 7 benzene acetic acid; 8 phthalic anhydride; 9 methyl parabens, 10 *p*-hydroxybenzoic acid.









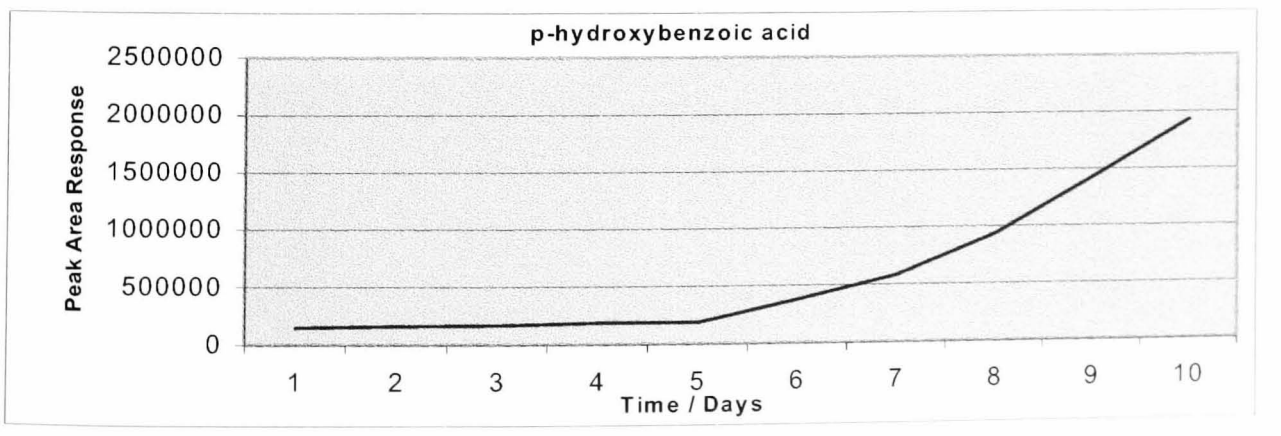
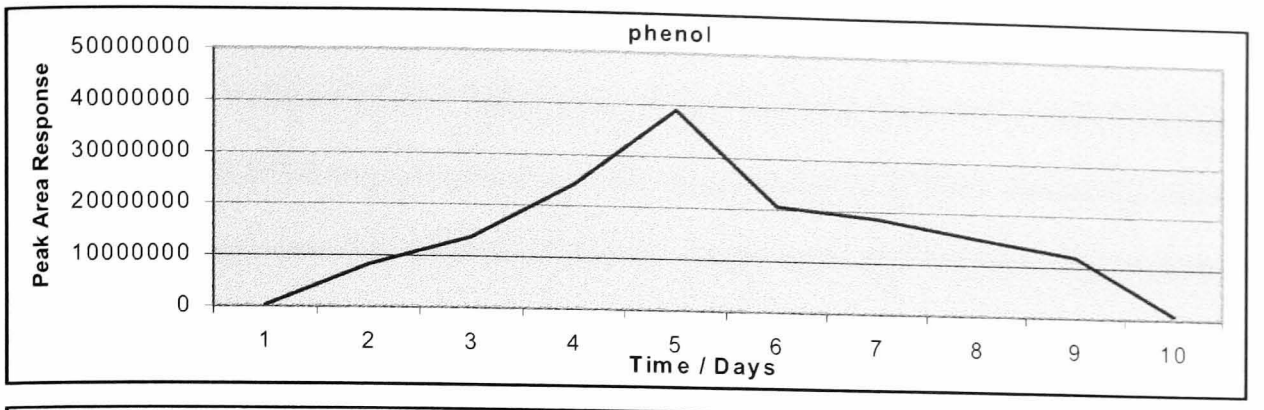
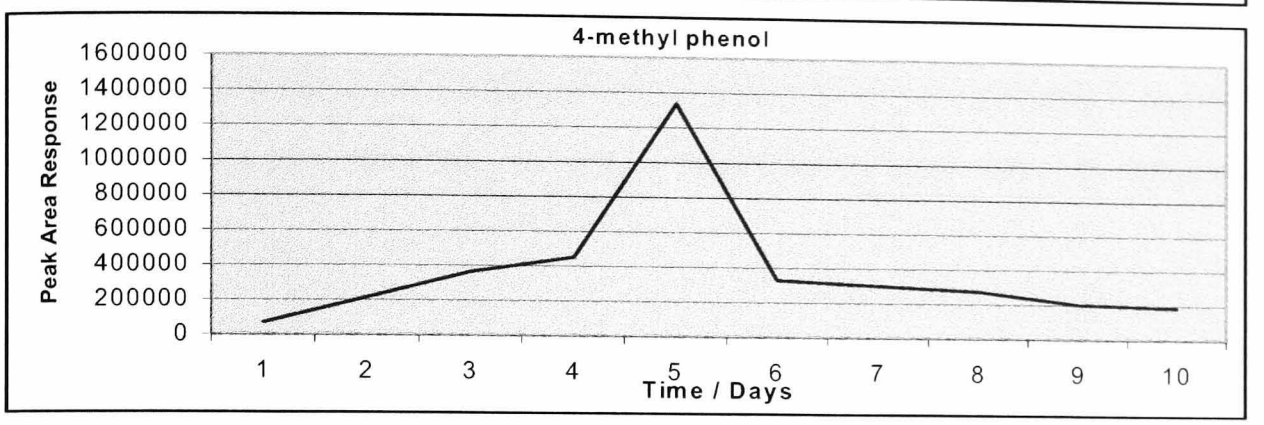
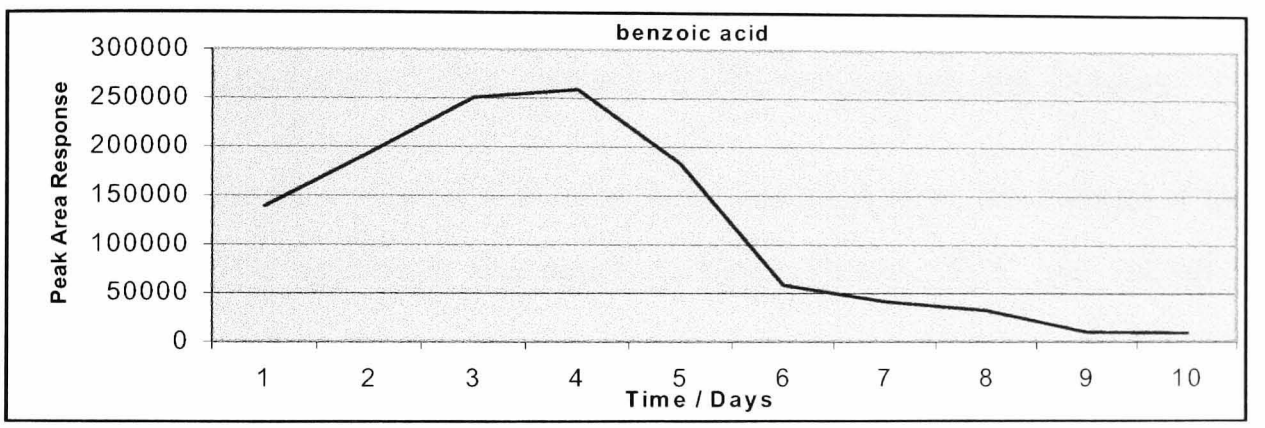
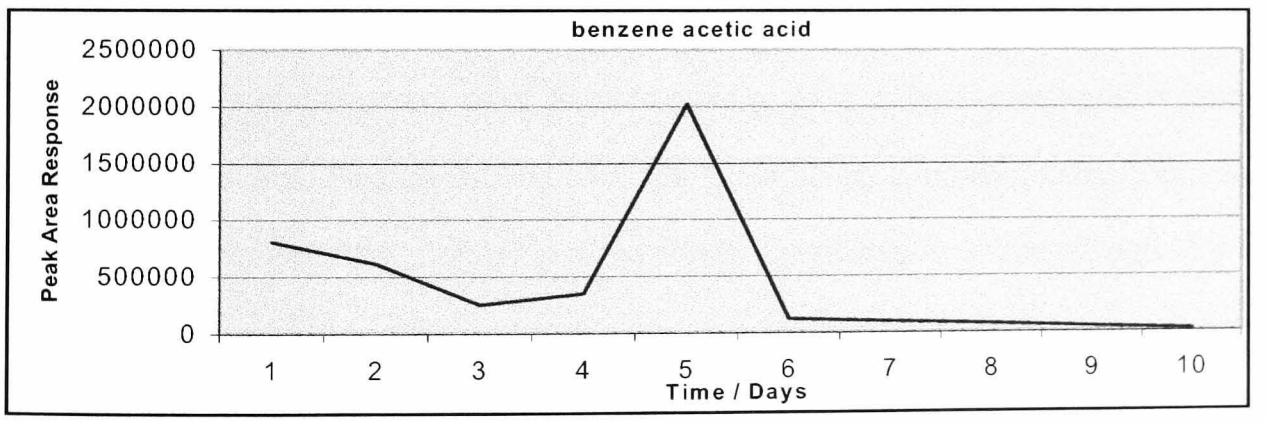


Figure 5.26 GCMS data charts for isovaleric acid, unknown polar compound, 2-ethylhexanoic acid, phthalic anhydride and *p*-hydroxybenzoic acid.









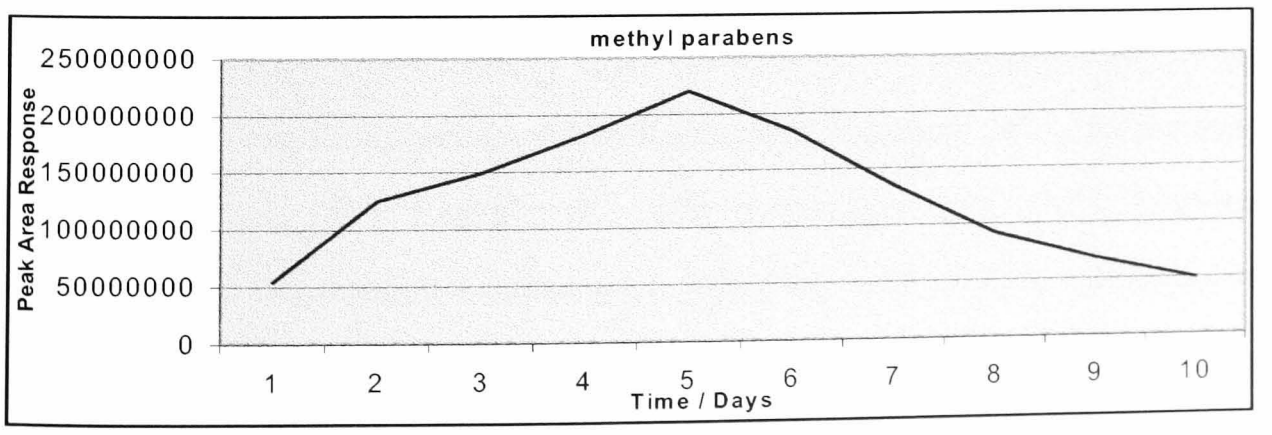


Figure 5.27 GCMS data charts for phenol, *p*-cresol, benzoic acid, benzene acetic acid and methyl parabens.

5.4.2 Discussion of PHBA degradation study

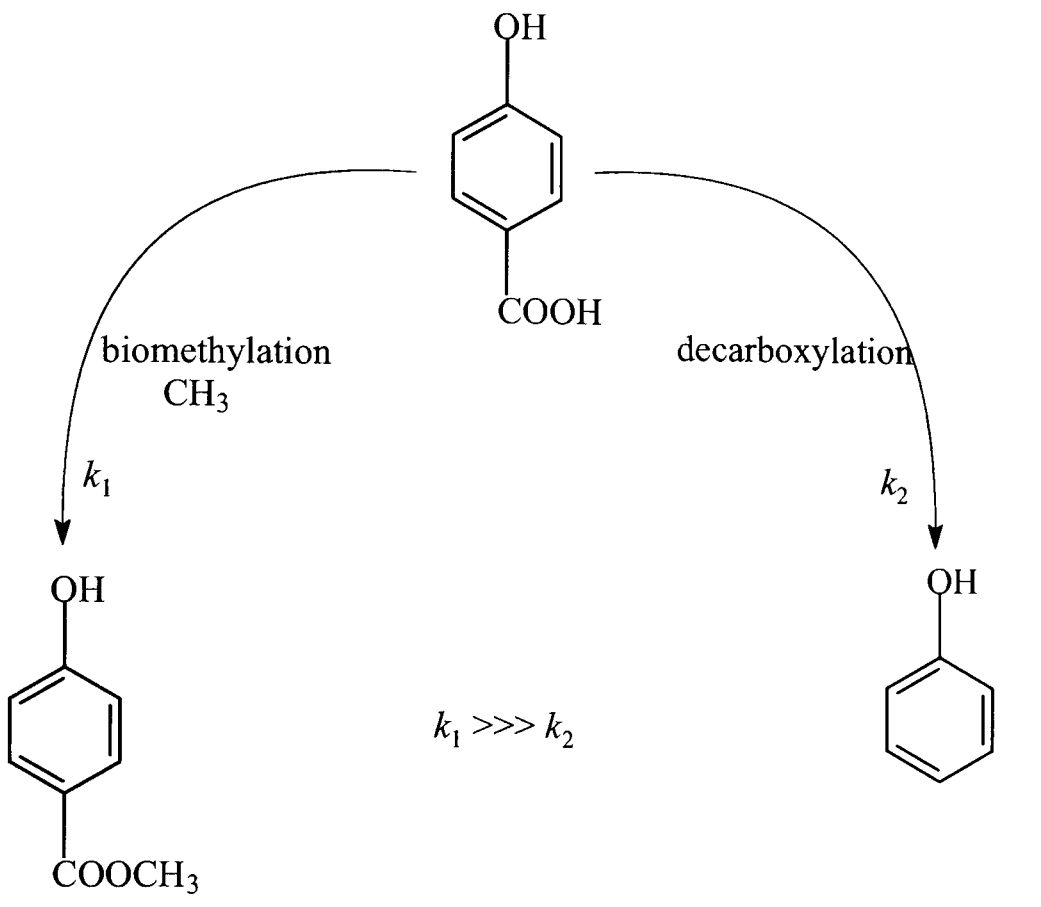
The initial observation of the behaviour of the biomass was that a fast methylation step precedes any other biological process in the degradation of PHBA. Biomethylation appears to be a dominant process whereby organic compounds are biologically broken down to simpler less toxic products, by the particular biomass at the Flexsys Ruabon site. In the majority of the studies carried out using the Ruabon biomass, biomethylation appears to be a common feature along the various pathways observed. Biomethylation is known to be a characteristic process observed in many biological systems used to treat industrial wastewaters.

This biomethylation process appears to be very robust and rapid, producing in this case a 99% conversion of the incoming substrate PHBA to the methylated ester based on an area percentage calculation from HPLC analysis of effluent collected after 24 hours.

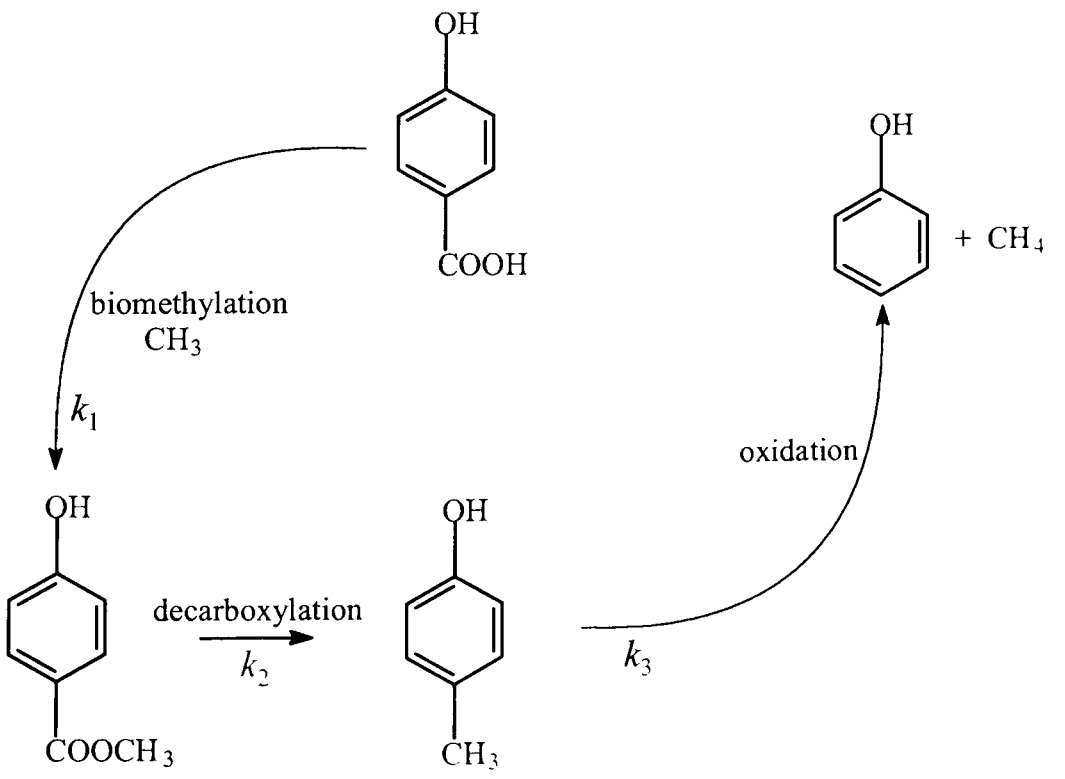
Analysis of samples collected after the initial inoculation of the biomass with a feed containing PHBA confirmed the above statement. Both HPLC and GCMS analysis revealed the presence of large amounts of methylated hydroxybenzoate (methyl parabens), with only traces of the parent substrate (PHBA).

There are numerous literature references reporting the fast biodegradation of phenols and phenolic compounds. Studies have shown that phenols are degraded in the following order under aerobic conditions: phenol and *p*-cresol, 3,4-xylenol, *o*-cresol and 2,5-xylenol, 2,6-xylenol, 3,5-xylenol. Hence, *para*-substituted phenols and phenol were first degraded and *meta*-substituted phenols were apparently the last to be degraded. Biodegradation of phenols by oxidation to catechols or hydroxybenzoates and subsequent ring cleavage has been reported (Feist and Hegeman, 1969; Hopper and Chapman, 1971; Hopper and Taylor, 1975; Baggi *et al.*, 1987; Ewers *et al.*, 1989; Hinteregger *et al.*, 1992; Powlowski and Shingler, 1994). Degradation via the catechol pathway is likely to be influenced by steric hindrance of hydroxylation by the methyl substituents on the aromatic ring. Hence, the order of degradation is likely to be influenced by the position of the methyl groups.

In the specific biomass used in this study of how PHBA is metabolised there appear to be two routes in operation, biomethylation and decarboxylation, with the methylation step preceding the decarboxylation step.



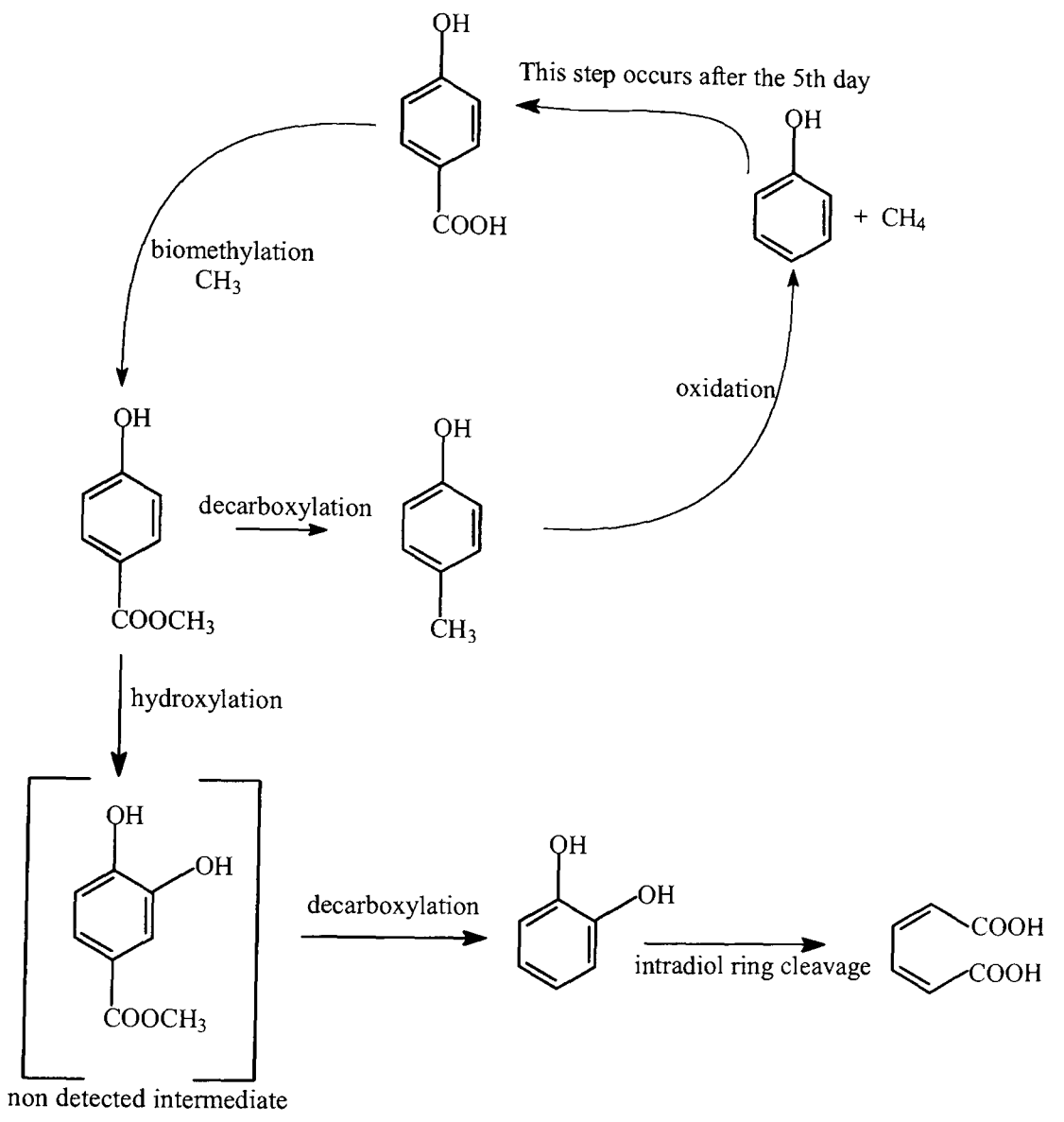
In the above scheme the pathway k_1 is very rapid and precedes the pathway k_2. However, the pathway k_2 may not actually occur as postulated above, but may come about via an alternative three step pathway *i.e.*



The above process leads to the formation of phenol from the methyl parabens generated in the initial step k_1.

The effluent from reactor R3 was found to contain significant levels of *p*-cresol, providing support for the possible route from methyl hydroxybenzoate (methyl parabens) to phenol via a decarboxylation step to the *p*-cresol metabolite.

The following metabolic pathway is proposed based on the sum analysis by HPLC and GCMS



An interesting development during the process was the generation of the metabolite isovaleric acid, initially identified by its characteristic odour. This compound had not been observed in any other experiments and was therefore assumed to be a metabolite along the degaradative pathway for PHBA, since in all previous experiments phenol has been present but not PHBA and no isovaleric acid had been detected. A further unexpected

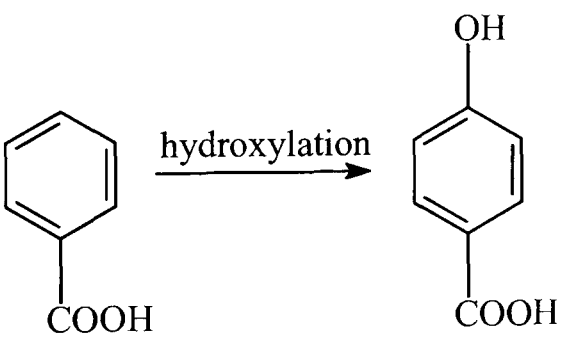
event was the apparent change in the metabolic pathway after five days with the regeneration of PHBA.

The majority of microorganisms that degrade phenol do so via the *ortho* cleavage pathway. For instance, of the 11 phenol oxidising *Pseudomonas* strains which Feist and Hegeman (1969) examined, eight strains degraded phenol through the *ortho* cleavage pathway.

Usually phenol degradation occurs via formation of the corresponding catechol which is then subsequently transformed by ring cleaving dioxygenases to muconic acid (Gibson and Subramanian, 1984; Wild *et al.*, 1996). The above conclusion is supported by the presence of both catechol and *cis,cis*-muconic acid in the effluent from reactor R3 during metabolisation of PHBA.

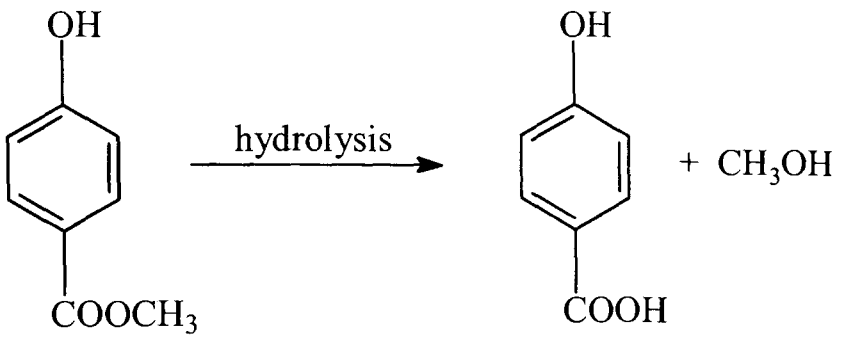
A further interesting development observed during the experiment was the increase in the PHBA concentration from day five onwards and for the remainder of the experiment. Possible mechanisms for this could be utilisation of phenol in an apparent backwards step of carboxylation, which although not an energy efficient mechanism, is feasible in principle.

A more plausible route however, may involve the hydroxylation of the metabolite benzoic acid.

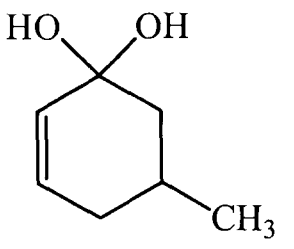


A further source of PHBA could be the breakdown/biotransformation of methyl parabens as part of the degradation pathway, this pathway being rate limited by the concentration of the PHBA present in the biomass (hypothesis 1), and once stimulated becomes the controlling mechanism for the removal of methyl parabens by a different bacterial strain to

give initially PHBA ⟶ methyl parabens (MP) followed later by MP ⟶ PHBA (hypothesis 2).



The structure (below) of the initially unidentified polar compound eluting early in both HPLC and the GCMS chromatograms was assigned on the basis of mass spectral analysis (Figure 5.28). This compound was the only other significant metabolite observed during the metabolisation of PHBA.



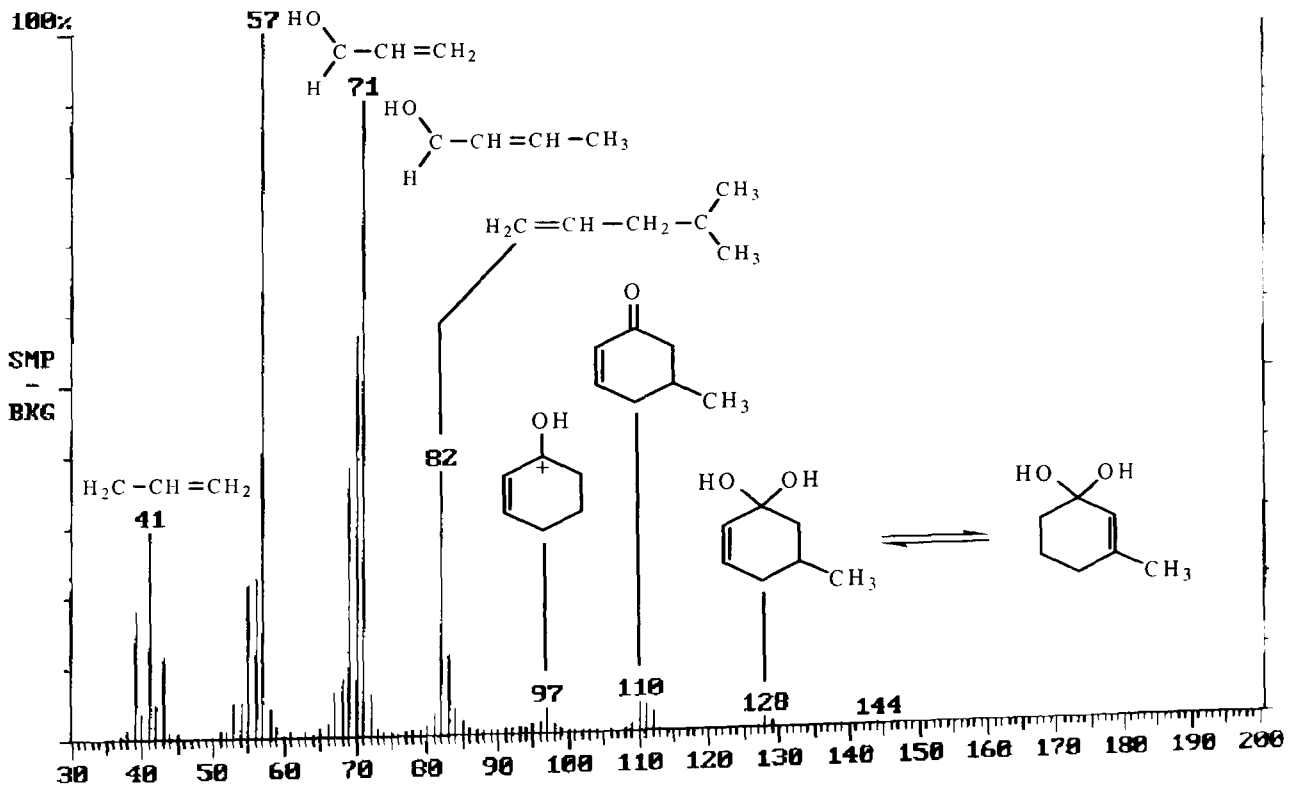


Figure 5.28 GCMS Spectrum for unidentified polar compound from PHBA study.

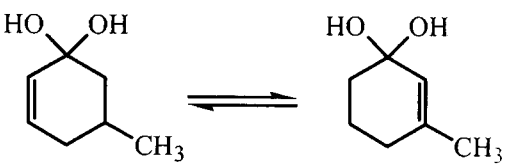
The structure elucidation for the unknown polar compound with an assigned formula weight of 128 (taken from the mass spectrum above) is given below.

Estimation of a molecular formula consistent with a FW of 128 is $C_7H_{12}O_2$

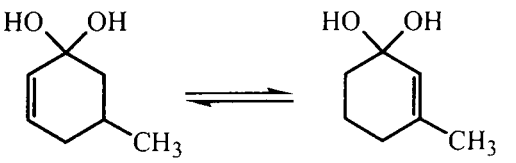
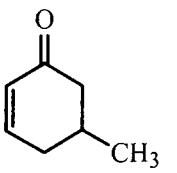
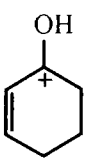
The number of double bond equivalents (DBE) value can be calculated using the formula $n - {}^1/_2x + 1$, where n = C and x = H (oxygen has having no effect on the DBE value).

For the molecular formula above, with $n = 7$ and $x = 12$ this gives $7 - {}^1/_2(12) + 1 = 2$. This could result from either a linear molecule containing two double bonds, one ring plus one double bond, or two rings (unlikely in this case).

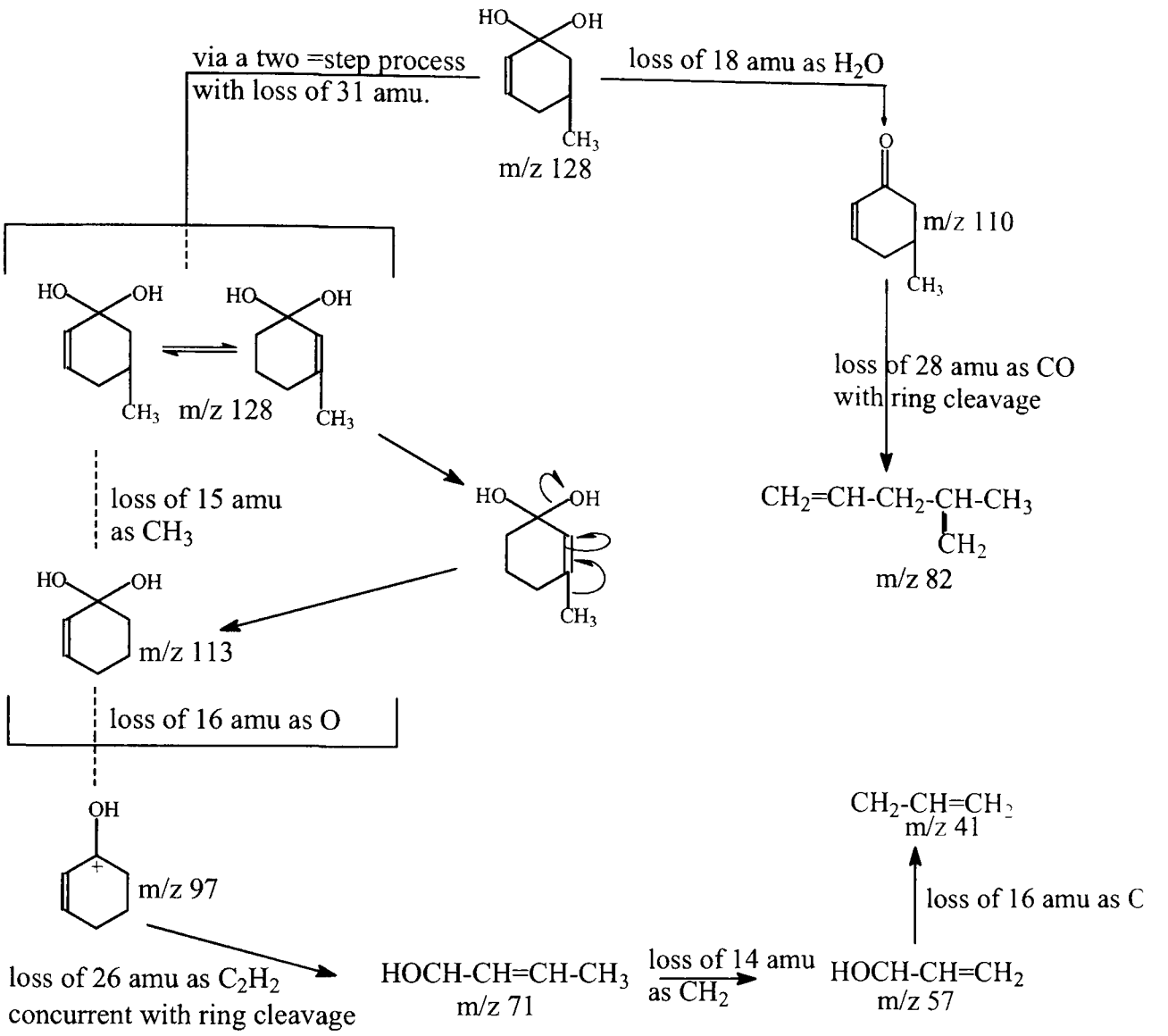
The following structure is consistent with a DBE value of 2.



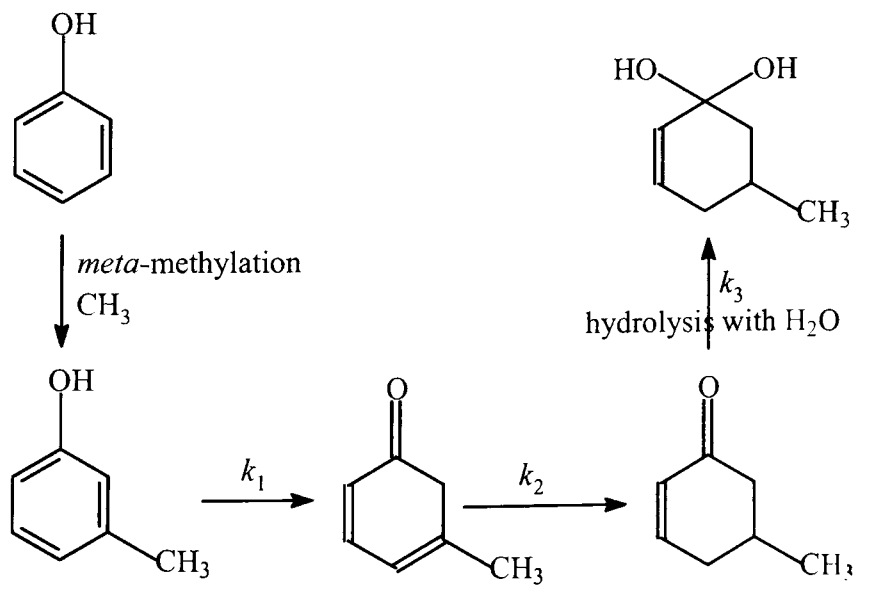
Working on the assumption that this is the structure of the compound in question then the following assignments could be made from the mass spectrum.

m/z value	fragment ion
128	
110	
97	
82	C_6H_{10}
71	C_4H_7O
57	C_3H_5O
41	C_3H_5

A possible fragmentation pathway for the above compound consistent with these assignments is as follows.



The polar compound identified above could be generated by the following mechanism using phenol as the initiating substrate shown below:



If k_3 is much faster than the sum of both k_1 and k_2 neither of the two intermediates would be observed in any analysis.

In order to investigate the apparent back reaction whereby PHBA is generated as a metabolite following inoculation of the biomass with PHBA as the feed substrate, it was decided to investigate the biodegradation of methyl parabens.

5.5 Biodegradation of methyl parabens

In a previous study investigating the biodegradation of *p*-hydroxybenzoic acid (PHBA), it was found that the most significant metabolite generated was the methyl ester of PHBA, methyl *p*-hydroxybenzoate, commonly referred to as methyl parabens. This is the first of a homologous series of alkyl esters of PHBA. Other important members of the series are ethyl, propyl and butyl parabens.

The alkyl parabens constitute one of the most important groups of chemical agents used as preservatives in pharmaceutical, cosmetic and other products. Although various studies of the homologous series of parabens esters have shown that their activity increases with increasing ester chain length (Hansch *et al.*, 1972; Eklund, 1985; Russell *et al.*, 1985; Russell and Gould, 1988); in practice the usefulness of the higher homologues is limited by their decreasing water solubility which in turn limits their bioavailability within the system.

The present study was concerned principally with confirmation of the observed reaction whereby methyl parabens was biotransformed to *p*-hydroxybenzoic acid within the biological system used at Flexsys Rubber Chemicals, Ruabon.

The biomass of reactor R3 was allowed to re-equilibrate with the base matrix synthetic feed over a two-week period to allow wash out of any traces of PHBA. Following this, once HPLC analysis had confirmed the absence of PHBA and associated metabolites, the biomass was inoculated with a feed consisting of the base matrix spiked with 100 mg/l methyl parabens over a ten-day period. All conditions were kept as for all previous experiments. Feed conditions were identical to those used for the PHBA study. Analysis of effluent samples was restricted to the application of HPLC for identification of residual methyl parabens and any generated metabolites.

5.5.1 Results of methyl parabens degradation study

The first 24 hour effluent sample, analysed by HPLC using method 8, confirmed the claim that methyl parabens could be a precursor to the generation of PHBA along the metabolic pathway for the removal of methyl parabens by the mixed consortia biomass used in this study (Figure 5.29)

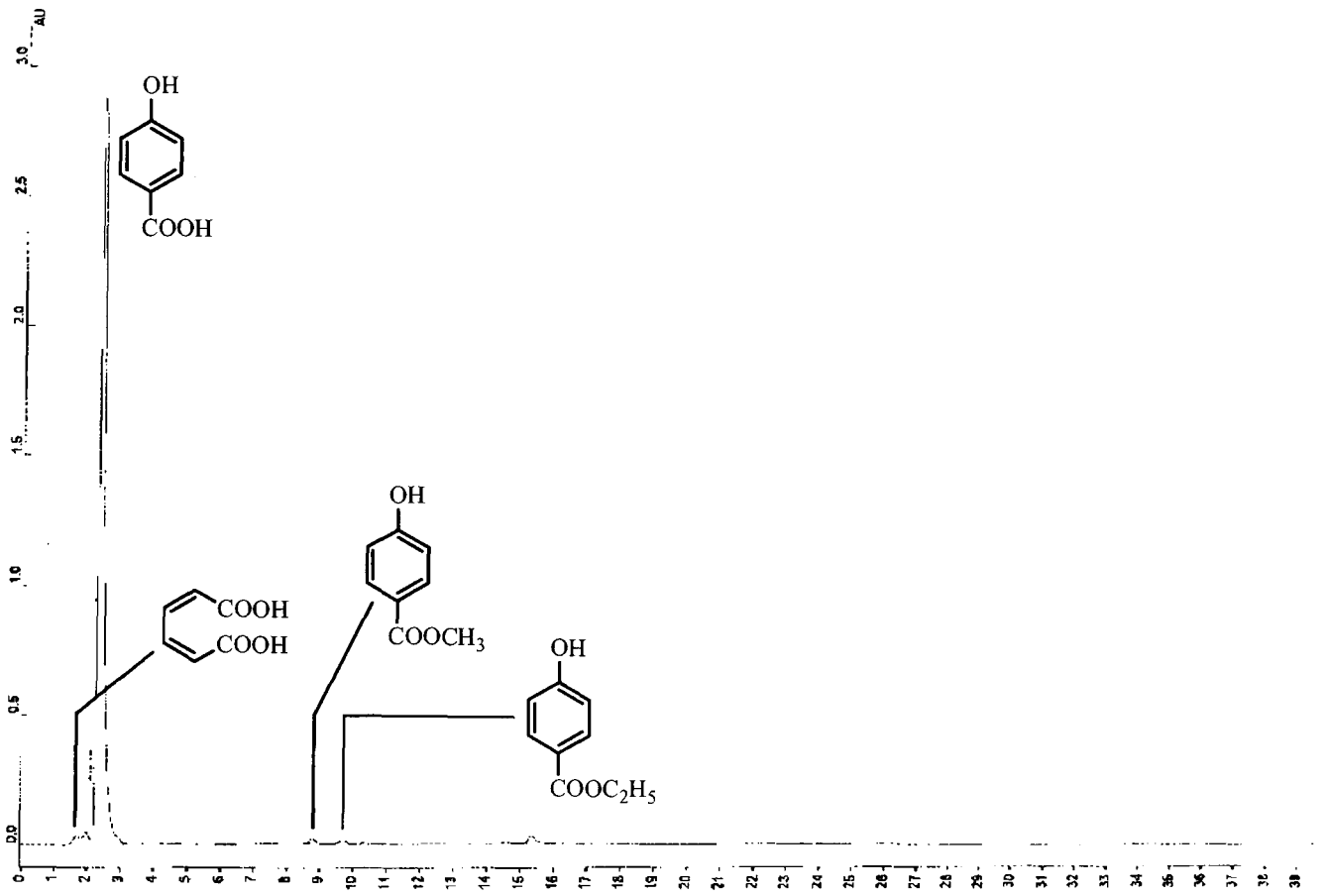


Figure 5.29 HPLC Methyl parabens the first 24 hours.

The chromatogram shows how quickly the incoming methyl parabens was biotransformed to principally PHBA with traces of *cis,cis*-muconic acid along with residual methyl and ethyl parabens. However, no phenol or catechol were detected within the first 24 hours of the metabolisation study. This also confirms previous literature claims that *para*-substituted phenols tend to be rapidly degraded under aerobic conditions.

The levels of PHBA was observed to increase with time over the whole of the experiment, along with other metabolites. By the end of day two (48 hours), a previously unobserved metabolite was identified – salicylic acid (*ortho*-hydroxybenzoic acid). The metabolite *cis,cis*-muconic acid continued to be generated by the biomass; however the expected metabolites phenol and catechol were still notably absent (Figure 5.30).

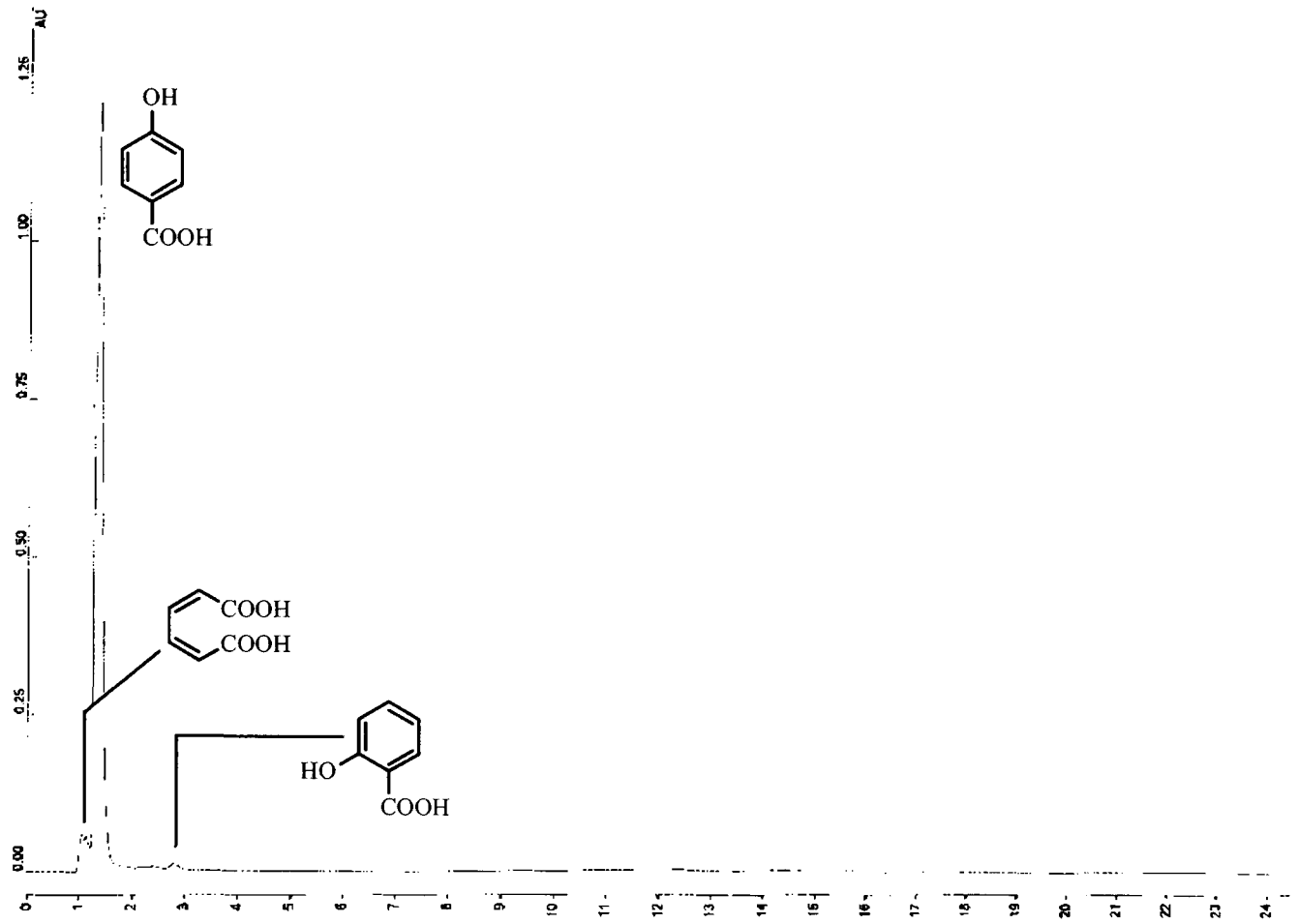


Figure 5.30 HPLC Methyl parabens 48 hours.

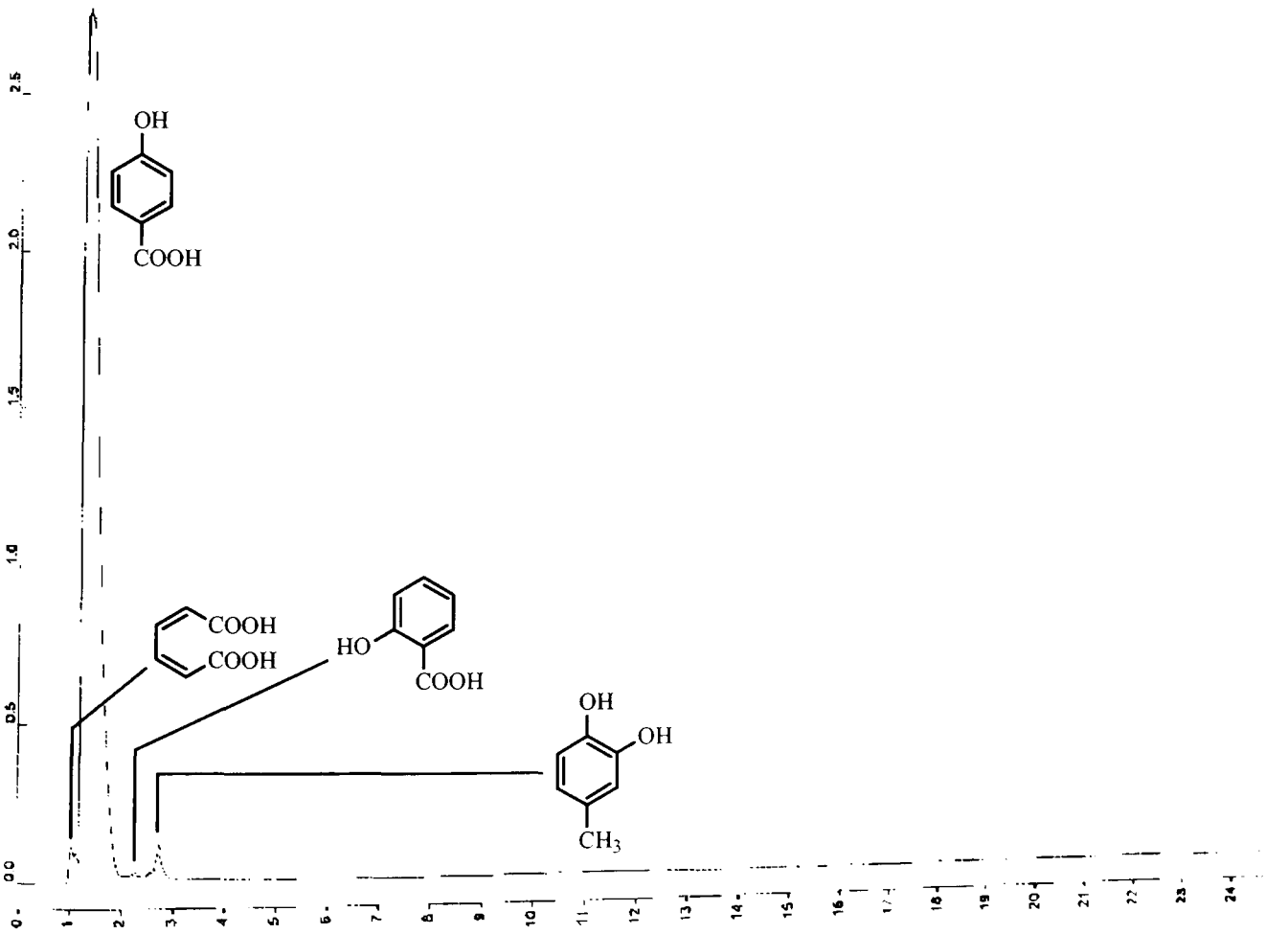


Figure 5.31 HPLC Methyl parabens 72 hours.

Analysis of effluent collected after 3 days (72 hours) by HPLC still showed no phenol or catechol to be present. However, a further new metabolite was identified, a methylcatechol (Figure 5.31).

Analysis of effluent collected over the remaining seven days of the study showed how the composition changed only in terms of metabolite concentration. Phenol was only detected in the effluent from day seven onwards at trace levels, the metabolite salicylic acid was not detectable in effluent samples from day eight onwards. The expected metabolite catechol was absent throughout the whole of the experiment. The HPLC data are plotted in Figure 5.32 and given in tabular form in Table 5.6.

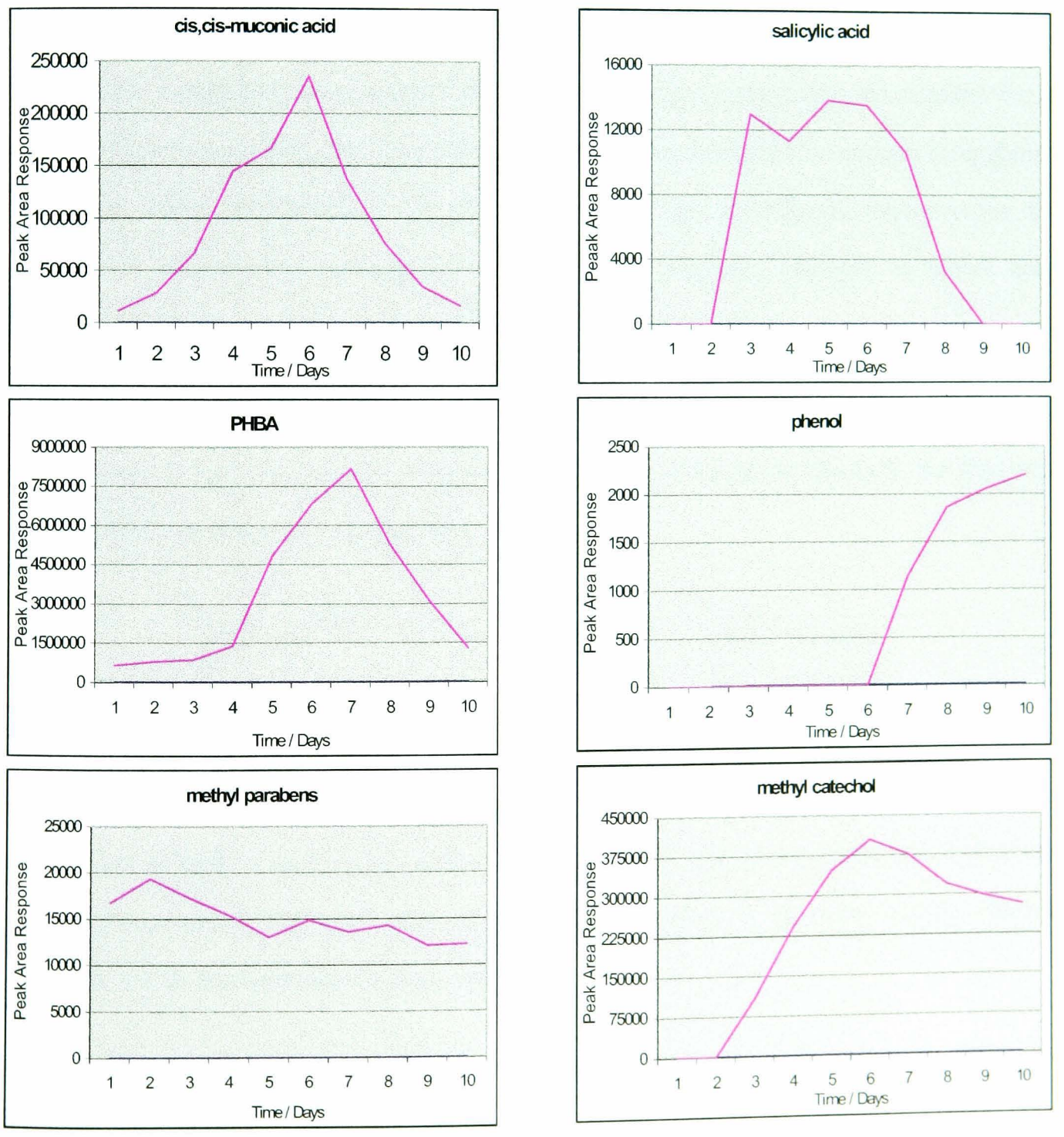


Figure 5.32 Peak area response charts for the data in Table 5.6.

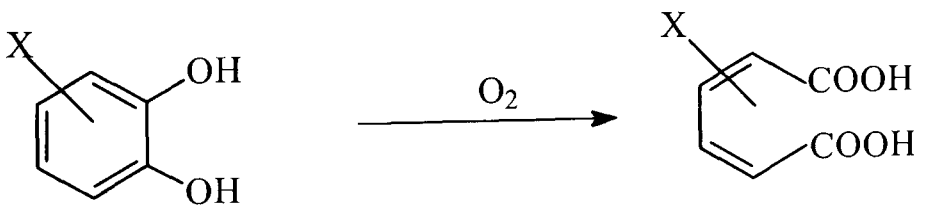
Table 5.6 Methyl parabens HPLC analysis peak area response data.

	Component ID/Peak Area Response					
Time/days	*cis,cis*-muconic acid	PHBA	salicylic acid	methylcatechol	phenol	methyl parabens
1	11582	648619	0.5	0.5	0.5	16753
2	28545	801618	0.5	0.5	0.5	19363
3	66160	864020	12982	108341	0.5	17286
4	144674	1385478	11321	242450	0.5	15411
5	166693	4832441	13847	347020	0.5	13058
6	235662	6845882	13545	404340	0.5	14867
7	137708	8170210	10634	376310	1136	13569
8	76361	5266340	3273	320544	1864	14253
9	34875	3125590	0.5	297955	2055	12098
10	16236	1296288	0.5	281077	2206	12247

5.5.2 Discussion of methyl parabens degradation study

The aerobic catabolism of aromatic compounds has been extensively investigated for a variety of microorganisms and for different natural and xenobiotic compounds (Häggblom and Valor, 1995). In particular many microorganisms use a catabolic sequence for the degradation of aromatic compounds called the β-ketoadipate pathway in which ring cleaving dioxygenases play a key role (Ornston and Stanier, 1966).

Among these enzymes, catechol 1,2-dioxygenases catalyse the intradiol cleavage of catechols to *cis,cis*-muconic acid with the incorporation of molecular oxygen, the first step in the overall sequence for catechol biotransformation.



There are a number of thermophilic bacteria that are capable of utilising aromatic compounds as carbon and energy sources. Thermophilic *Bacillus* isolates which degrade phenol, benzoate, cresol and other substituted aromatics have been widely studied (Buswell, 1974; Buswell and Thomey, 1975).

Cleavage of the aromatic ring is typically achieved via the *ortho*- or *meta*-pathways (Ornston and Stanier, 1966), the latter generally observed in thermophilic bacilli. The degradation of phenol by *meta*-cleavage utilises a sequence of (a) hydroxylation to

catechol, (b) ring cleavage via catechol-2,3-dioxygenase to 2-hydroxymuconic semi-aldehyde, and (c) either oxidation to 4-oxalocrotonate or hydrolysis to 2-oxopent-4-enoate.

A number of possible biotechnological applications of aromatic-degrading organisms and their constituent enzymes have been investigated included the use in bioreactor systems for removal of toxic waste products or treatment of contaminated waste streams (Collins and Daigulis, 1997).

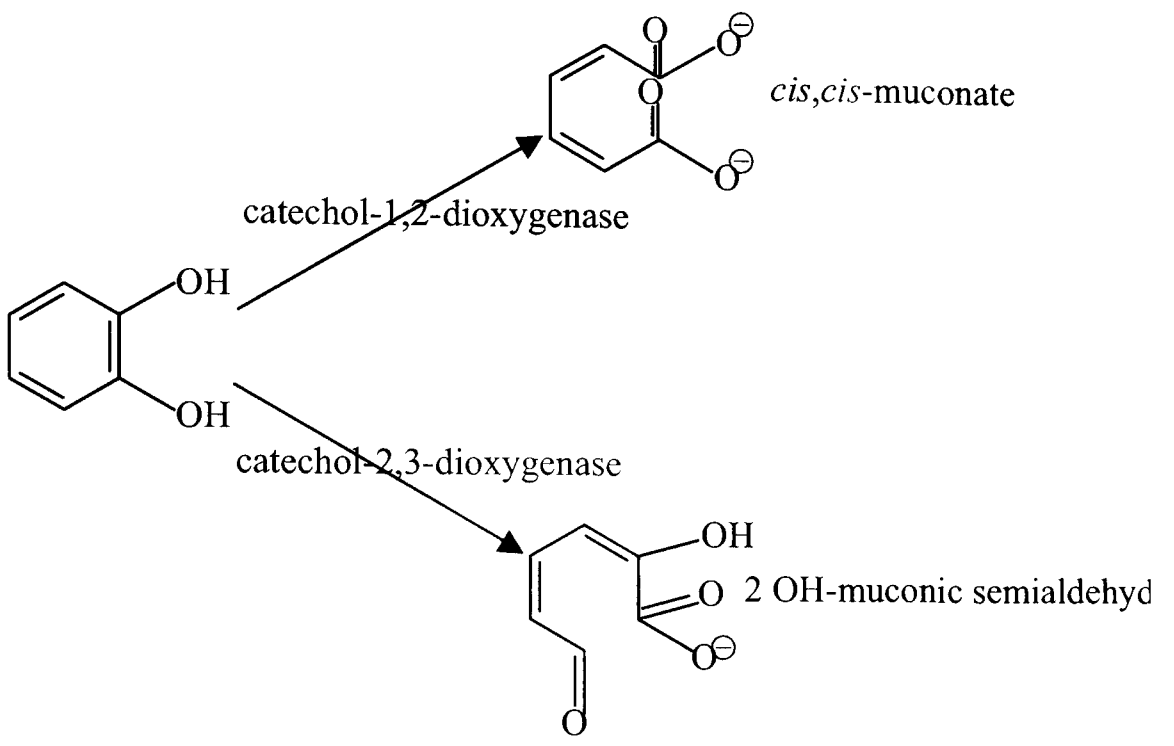
In the previous experiment *p*-hydroxybenzoic acid (PHBA) was degraded by a mixed consortia of bacteria under aerobic conditions via biotransformation to methyl parabens. The forward reaction PHBA ⟶ methyl parabens was subsequently observed to reverse with the regeneration of the parent substrate PHBA from day five onwards of the experiment.

In this study methyl parabens was fed to the same mixed consortia to stimulate the observed pathway methyl parabens ⟶ PHBA. Analysis of resulting effluent samples taken from the system following inoculation with methyl parabens confirmed that a metabolic pathway exits whereby methyl parabens is biotransformed into PHBA. In both experiments a similar suite of metabolites was generated by the biomass showing the inter-relation of the two substrates and how they induce enzyme production.

Phenol and *cis,cis*-muconic acid were generated as metabolites along the degradative pathways; however the metabolite methylcatechol was a metabolite along the methyl parabens pathway only. The catechol can be further degraded via two pathways, *ortho*- or *meta*-cleavage. Catechol is one of the 'common intermediates' to which all aromatic compounds get funnelled (Hamzah and Al-Baharna, 1994; Harwood and Parales, 1996).

Ortho-cleavage pathway utilising catechol-1,2-dioxygenase transforms catechol to *cis,cis*-muconic acid, and the acid is converted to a lactone (internal ester) by addition of a carboxylic OH across one of the double bonds.

Meta-cleavage pathway utilising catechol-2,3-dioxygenase transforms catechol to 2-hydroxymuconic semialdehyde or analogue. This carbonyl compound is hydrolysed to a simple organic acid and an α-keto-ω-unsaturated acid.



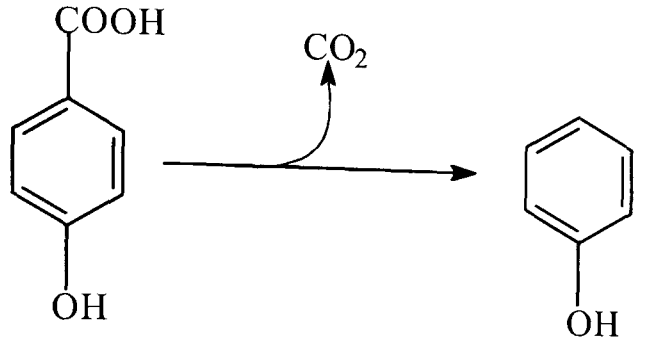
These two pathways sometimes occur within the same organism. Sometimes it is the source of catechol that determines whether the catechol is degraded via *ortho-* or *meta-* cleavage. Catechol formed from benzoate is degraded via the *ortho-* pathway, catechol formed from phenol is degraded via the *meta-* pathway.

The presence of *cis,cis*-muconic acid in both experiments (*i.e.* PHBA and methyl parabens biodegradation) would suggest a common precursor for the production of the muconic acid. In the case of PHBA it would appear that formation of phenol must precede the production of *cis,cis*-muconic acid whereas *cis,cis*-muconic acid is a metabolite of methyl parabens degradation.

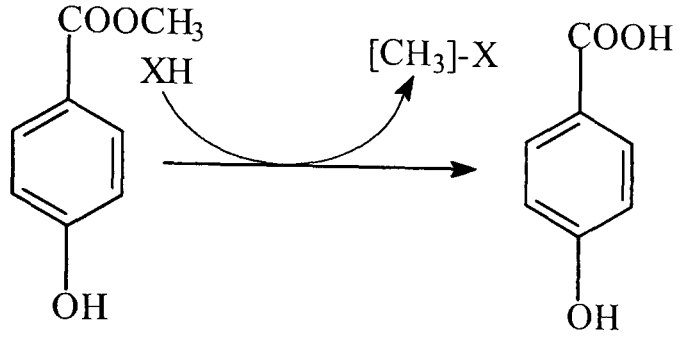
Carboxydiols are known to be very unstable compounds: in fact they undergo acidic decomposition, giving rise to two kinds of products, phenols and/or salicylic acids. In the work of Schmitt *et al.* (1984) the diol from benzoate gave both phenol and salicylic acid.

The following pathways are proposed on the basis of findings from both PHBA and methyl parabens biodegradation studies using a mixed consortia aerobic biomass.

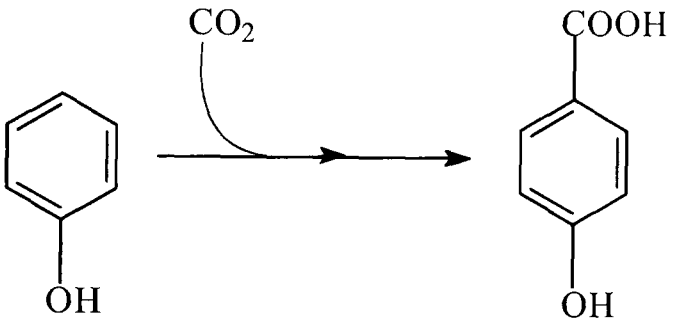
Production of phenol from PHBA without oxygen: via a decarboxylation step.



Production of PHBA from methyl parabens: via a demethylation step.

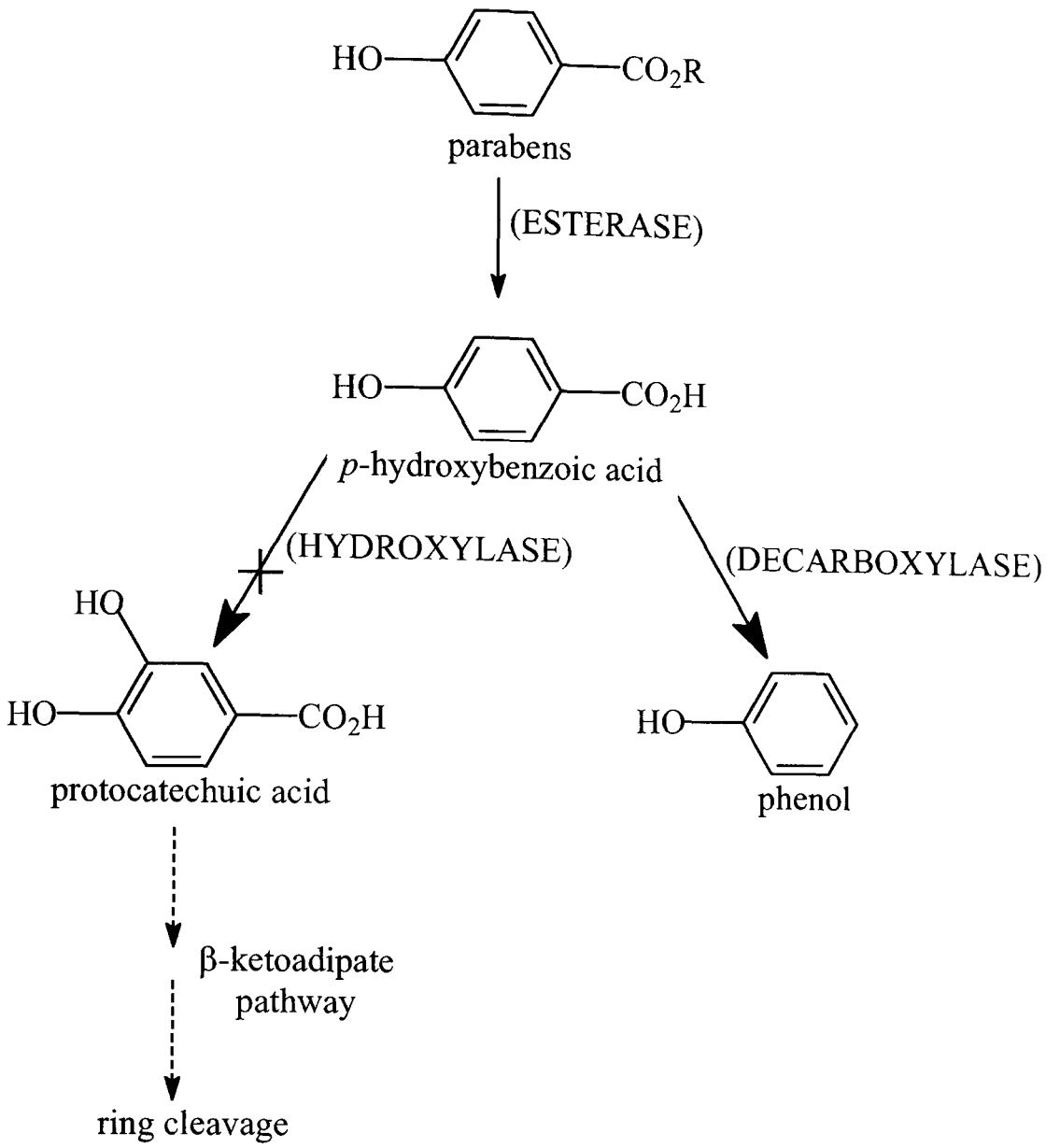


Carboxylation of phenol to yield PHBA.



Similar findings have been reported by other workers in the field studying the biotransformation of both PHBA and associated esters. Valkova *et al.* (2001) reported that PHBA was biotransformed into phenol via a decarboxylation step. According to Valkova *et al.* this decarboxylation of *p*-hydroxybenzoic acid into phenol by aerobic bacteria has previously only been reported once using a strain of *E. aerogenes* (Steigerwatt *et al.*, 1976) under both aerobic (Patel and Grant, 1969) and anaerobic conditions (Grant and Patel, 1969).

Valkova *et al.* proposed the following mechanisms for the biotransformation of *p*-hydroxybenzoic acid.

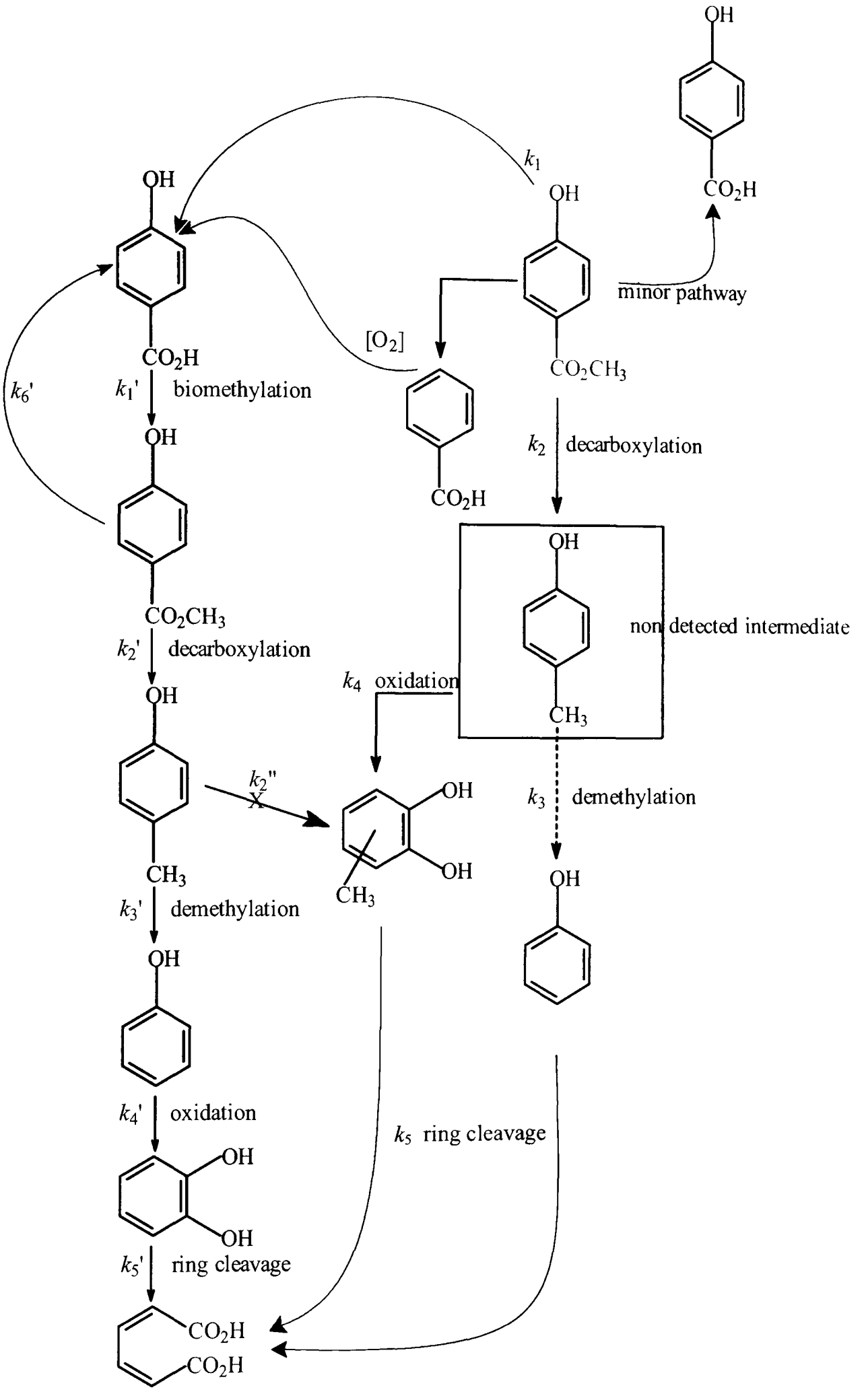


The usual degradation pathway of PHBA by aerobic bacteria is through the β-ketoadipate pathway, resulting in the formation of protocatechuic acid instead of phenol. However, Valkova and co workers reported that under the conditions of their study no protocatechuic acid was detected by either HPLC or GCMS analysis. Rather they reported a stoichiometric conversion to phenol. It has been reported that under anaerobic conditions that PHBA can be decarboxylated into phenol (Grant and Patel, 1969). However the aerobic transformation of PHBA into phenol is a rarely documented pathway and raises questions about the ability of other ubiquitous *Enterobacteriaceae* to carry out these reactions.

The above pathway was also observed in this study during the aerobic biodegradation of PHBA and methyl parabens by a mixed consortia of bacteria. Analysis of effluent samples taken during the course of the study enabled the following pathways to be proposed (overleaf).

Key: PHBA as sole substrate

Methyl parabens as sole substrate



In both pathways the expected metabolite protocatechuic acid is absent confirming that an alternative to the β-ketoadipate pathway exists whereby both PHBA and methyl parabens give rise to phenol and *cis,cis*-muconic acid. The second experiment, that of methyl parabens biodegradation, also confirmed the observed concentration limiting step of the biotransformation from methyl parabens to PHBA. The step k_6' is clearly concentration dependent since it does not occur until day five of the PHBA degradation study whereas the transformation of methyl parabens to PHBA is very rapid when methyl parabens is the sole substrate source. Two points to note are: (a) cresol is either not generated out of methyl parabens or the biotransformation to phenol and/or methylcatechol is so rapid that the species is not detected; (b) methylcatechol is not detected along the metabolic pathway for PHBA. Both these points provide further supporting evidence for the existence of differing metabolic pathways for the same substrate depending on its initial source.

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CHAPTER 6
THE 'RED COLOUR' PHENOMENON

6.1 Introduction

The original wastewater treatment plant (WWTP) layout is shown in Figure 6.1. This plant design consistently produced a yellow coloured effluent. However, there were two occasions when the effluent turned red, one in the early 1960s the second in the late 1960s.

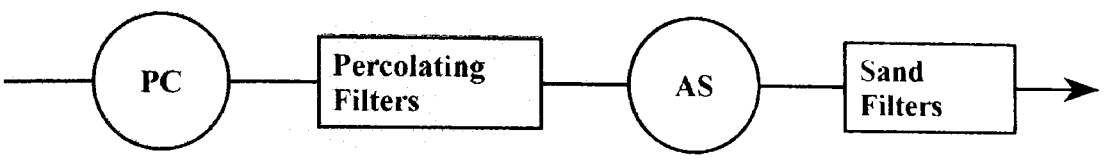


Figure 6.1 The original layout of the wastewater treatment plant.
PC primary clarifier; AS activated sludge bioreactors.

The plant was subsequently modified in 1985 by uprating the aeration system to make use of the BOC Vitox pure oxygen injection system. At the same time the percolating filters were removed (Figure 6.2).

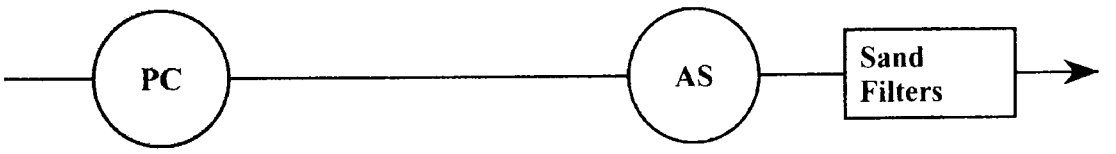


Figure 6.2 The layout of the wastewater treatment plant following the 1985 modifications.

Following the modifications to the original design the effluent was consistently red/pink in colour. The plant was further modified in 1990 with the addition of a second activated sludge bioreactor and settling tanks; at this point the final sand filters were also removed from the plant (Figure 6.3).

A widely-used measure of water quality is the total organic carbon (TOC) content of the treated water, a high value indicating poor quality and treatment. However, to the layperson, colour is an important indicator of water quality. If a body of water is highly coloured it is often interpreted as being quite polluted (Brown and De Vito, 1993; Pierce, 1994; Knapp and Newby, 1998; Kang and Kuo, 1999).

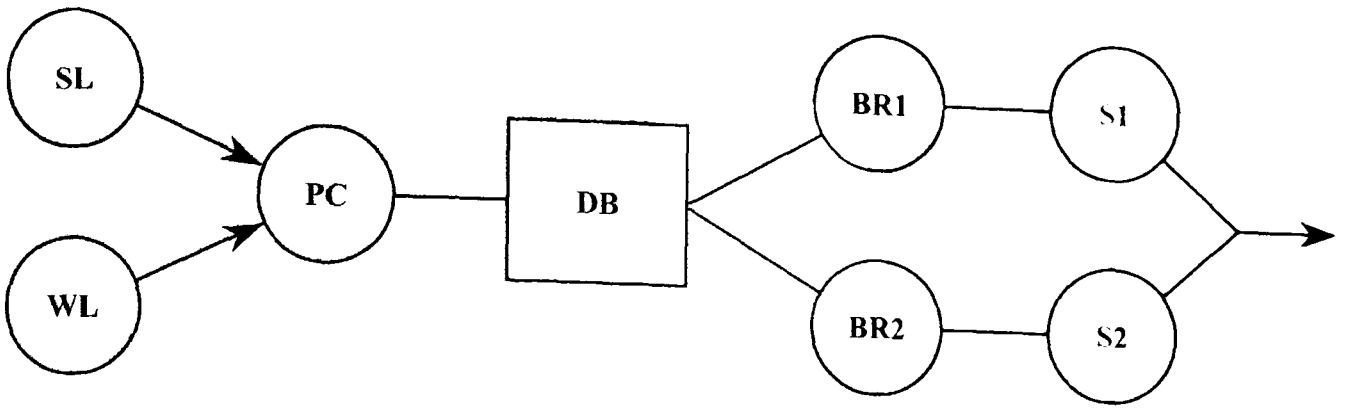


Figure 6.3 The final layout of the wastewater treatment plant. Key: PC primary clarifier; SL strong liquor from manufacturing plants; WL weak liquor from manufacturing plants; DB distribution box; BR1 and BR2 bioreactors 1 and 2; S1 and S2 settlers 1 and 2.

This is often not the case as with the treated effluent from the Flexsys Ruabon manufacturing site. As stated in Chapter 1, on occasion the treated water from the biological treatment plant is highly coloured and to the layperson this could be an indication of gross pollution and/or poor treatment. The reality is far from the truth in this case.

Considering the analysis parameter TOC as being a good indicator of water quality, the lower the TOC value the better is the quality of the treated water in terms of dissolved non-specific organic compounds. Analysis of the treated effluent leaving the Flexsys Ruabon site wastewater treatment plant during periods of changing colour in the effluent was as shown in Table 6.1.

Table 6.1 Relationship between colour of effluent and TOC values.

Colour of Effluent	**mg/l TOC**
Colourless	18–20
Pale straw	18–20
Yellow/amber	18–20
Pink	18–20
Intense red	18–20

The above results show how little the quality of the treated waters varies with colour intensity. The TOC change during periods of high and low colour is so small that it is not detectable even by the most sensitive test procedure. It does however indicate that the 'red' chromophore has a very high molar absorption coefficient.

It was thought that this phenomenon was unique to the Flexsys Ruabon site. However, it has recently (June 1999) become known that two other Flexsys locations also have a similar red problem with their effluent, both sites also utilising biological treatment facilities. A third water treatment plant, in Akron, Ohio, USA also utilises the activated sludge process but this plant does not produce a red coloured effluent. This plant is also different from the others in that it is a low efficiency plant in terms of carbon removal. Furthermore, other researchers investigating the use of activated sludge to treat thiazolic wastewater also observed a red coloured effluent from the system (Liu *et al.*, 1983; De Vos, 1993b).

Over the years a lot of circumstantial evidence has been collected which links the colour problem to the presence of 2-mercaptobenzothiazole (MBT). De Wever (1995) conducted experiments to determine the conditions that favoured the formation of the substance containing the 'red' chromophore. The findings indicated that combinations of benzothiazole (BTH) and 2-hydroxybenzothiazole (BTOH) give rise to a red coloured effluent from an activated sludge system.

Reviewing data collected during periods of intense colour production, it was found that high red colour tended to develop when the following conditions prevailed:

- High sludge blanket and long settler residence times – conditions becoming more anaerobic than normal.
- On many occasions when high 2-methylmercaptobenzothiazole (MeMBT) levels were detected (*ca.* 600 µg/l) *vs* normal levels of 23 to 38 µg/l.
- Analysis of water samples taken from the settlers during colour changes:
  when yellow – benzothiazole-2-sulphonic acid (BTSA) < 0.1 µg/l
  when red – benzothiazole-2-sulphonic acid (BTSA) levels very high ~ 200 µg/l.

It had also been observed that the red colour of the effluent increased following a period of high MBT levels in the feed to the biomass. However, close examination of recorded data for MBT levels in the feed also showed that periods of high colour followed low MBT levels in the feed. On occasion the red colour would be negligible even following high levels of MBT in the feed.

Essentially the cause of the red colour was not known though linked to the presence of MBT. This is also supported by the findings of De Wever (1995). Liu *et al.* (1983) also made reference to the production of a substance with a yellow chromophore during the biological degradation of thiazolic wastewaters.

It would appear that the colour production is greatest in the sludge thickener stage of the treatment process, which would suggest that an oxygen deficient (anoxic) environment favours the formation of the red chromophore. To confirm this hypothesis, samples of biomass from an oxygen-rich environment were taken and put in an airtight container and placed on a window shelf alongside the laboratory biological reactor system. Within 24 hours the supernatant above the settled sludge had turned red. It was noted also that during the day, while the sample was left on the window shelf being exposed to sunlight, the colour began to quickly fade.

The experiment was repeated but this time the container was placed in the dark inside a cupboard overnight. The following morning the solution above the settled sludge had again turned red. Leaving the container in the dark for a further 24 hours, with occasional shaking, did not cause the colour to fade as before. The container was then placed in direct sunlight and the colour faded in a similar manner to the first experiment. This confirms that the chromophore is photosensitive.

In order to maximise the chances of isolating the product containing the chromophore, samples were taken from the sludge thickener for use in isolation studies. These samples were stored in the dark to prevent any photochemical degradation of the colour.

6.2 Solubility studies of the 'red chromophore'

A series of 300 cm^3 samples of sludge from the thickener were extracted with a range of solvents. The results are shown in Table 6.2.

Table 6.2 Results of extraction of sludge with various solvents.

Solvent	Result
Toluene	no appreciable colour extraction into the organic phase
Chloroform	no colour extracted into the organic phase
Methanol	some colour extraction into the organic phase
Acetone	good colour extraction into organic phase
Acetone/Water (90:10)	strongest colour extraction in the organic phase.

6.3 Isolation of 'red chromophore'

A two-litre sample of sludge was obtained from the sludge thickener and was filtered using a Buchner funnel to reduce its water content. The resulting filter cake was then washed with 500 cm^3 acetone, the acetone extract filtered to remove the sludge and the procedure repeated using a fresh 500 cm^3 aliquot of acetone. The two acetone extracts were combined and placed in a pre-weighed Buchi rotovap flask. The acetone was slowly distilled to yield a thick oily red residue of mass 0.35 mg.

6.4 TLC studies

TLC analysis was applied to the acetone/water extract to determine if the colour was composed of more than one chromophore. The separations were carried out using Kodak 70 × 30 mm size TLC silica plates. A single spot of the acetone/water extract was applied to a series of five plates. Each plate was developed with the results shown in Table 6.3.

Table 6.3 Results of TLC analysis of acetone/water extract of sludge.

Solvent	Result
Dichloromethane	spot did not move from origin (but a very faint yellow coloured spot was just observable at the solvent front)
Ethyl acetate	same as above for dichloromethane
Acetone	brown spot at solvent front, red streak from origin to solvent front. However, most of the colour still remained at the origin
Acetone/Water (90:10)	red spot moved with solvent front, almost no colour remained at origin
Acetone/Toluene (10:90)	no movement of spot from origin

These TLC studies seem to suggest the presence of a second coloured component. It would also appear that both coloured products posses polar characteristics, although the yellow component shows some solubility in dichloromethane and ethyl acetate, suggesting that it is probably less polar than the red substance.

6.5 UV/visible spectroscopic analysis of isolate

The acetone/water extract was diluted with distilled water to produce a solution, which on analysis gave rise to a spectrum having an absorption maximum at 330 nm with a second less intense absorption in the visible region at 520 nm.

Analysis of the various thiazole products showed that 2-mercaptobenzothiazole (MBT) has a λ_{max} at 310 nm. From this it may be speculated that the red substance may have structural similarities to MBT.

6.6 Solid phase extraction studies

Various sorbents were used in an attempt to characterise the nature of the coloured product present in the effluent. In all experiments 250 cm^3 aliquots of coloured filtered effluent were used. After filtering to remove solids, which otherwise would block the solid phase extraction (SPE) cartridge, the samples were adjusted with strong hydrochloric acid to a pH of 2.0. The solutions were also 'salted' out by addition of 10% w/v sodium chloride. The extraction rates were adjusted to give an extraction time of 15 minutes for a 250 cm^3 sample volume.

All the SPE cartridges were preconditioned as described in Chapter 2, Section 2.3.2. Sorbents used to extract the coloured component were Isolute™ PRS, Isolute™ NH_2, Isolute™ SAX and Isolute™ ENV+ (the name 'Isolute' is the trade name of International Sorbent Technology (IST), the manufacturer of the various SPE cartridges used in the study of colour extraction).

6.7 Results and discussion of SPE studies

6.7.1 Isolute ENV+

This sorbent is a hyper cross-linked styrene-divinylbenzene copolymer which is able to effectively extract many polar compounds. When using this sorbent all the colour of the aqueous sample was retained in a tight band at the top of the sorbent bed. This would imply that the isolate is a polar compound.

6.7.2 Isolute PRS, Isolute NH_2 and Isolute SAX sorbents

These sorbents represent the range of ionic sorbents available. Isolute PRS (Figure 6.1a) is a strong cation exchanger, which is used to extract positively charged basic compounds. By comparison Isolute NH_2 (Figure 6.1b) is a dual purpose sorbent that can act as both a polar phase and a weak anion exchanger. When used in an aqueous environment at pH values below 7.8, this sorbent can function as a weak anion exchanger because all the NH_2 groups are protonated and the column can retain molecules that carry a negative charge. The Isolute SAX sorbent (figure 6.1c) is a strong anion exchanger, which is ideally suited to the isolation of weak acids.

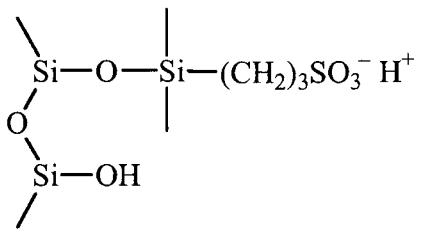


Figure 6.1a Chemical structure of the PRS silane, covalently bonded to the surface of a silica particle.

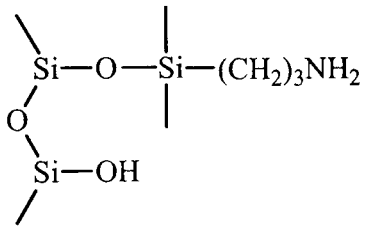


Figure 6.1b Chemical structure of NH_2 silane, covalently bonded to the surface of a silica particle.

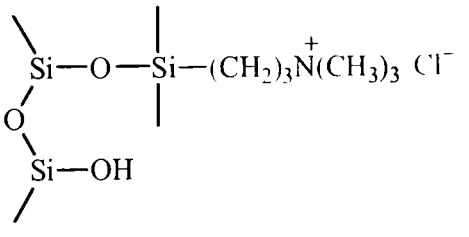


Figure 6.1c Chemical structure of SAX silane, covalently bonded to the surface of a silica particle.

There was no colour retention on the Isolute PRS sorbent, from which it is possible to infer that at low pH the red product carries a negative charge. The Isolute SAX sorbent trapped the colour in a single tight band at the top of the sorbent bed. Using the Isolute NH_2 sorbent two bands were observed. A red band retained at the top of the sorbent bed with a second yellow band observed halfway down the column.

The results from both the Isolute SAX and Isolute NH_2 columns would suggest that the molecule is negatively charged at low pH. Furthermore, the red product appears to carry a

stronger charge compared to the yellow product. From this it is possible to postulate that the red substance may well contain an SO_3^- functional group within its structure.

6.8 Colour production in laboratory bioreactors

The colour of the effluent from the three laboratory reactors varied between a very pale straw yellow to a dark yellow/amber, but was never red. The colour tended to be predominantly yellow, even when the biomass was exposed to high levels of various benzothiazoles. This is in contradiction to the findings of De Wever (1995), who observed the production of a red colour during biodegradation studies of benzothiazoles. However, on one occasion the colour of two of the three settlers turned red for no immediately apparent cause which presented an ideal opportunity for study.

6.8.1 Laboratory biological reactor colour

The following sequence of events took place leading up to the production of colour within the laboratory reactors.

On day one (a Friday), a volume of feed was obtained from the main wastewater treatment plant (WWTP) to maintain feed over the weekend for reactors R1 and R2. Also on day one, a larger volume of feed was obtained from the WWTP for a fourth biological reactor system (owned and managed by the Flexsys Process Engineering Group (PEG)). The volume was such that no more feed would be required until the following Wednesday (day six). Fresh feed was obtained for reactors R1 and R2 on the Monday morning (day four).

By the morning of day five the colour of both settlers for reactors R1 and R2 had turned red which continued to develop during the rest of the day. The colour had however completely gone by 9 am the next morning (day six).

The PEG reactor feed aspirator was restocked with fresh feed from the WWTP on the morning of day six. Within 24 hours the settler unit for this reactor had also turned red However, the colour in this unit persisted for between 24 and 48 hours.

Analysis of retained samples for the various feeds showed that certain compounds were missing from the usual matrix in the samples from the WWTP taken on day one. These

same components were found to be present in the day four and day six samples of feed from the WWTP.

The components missing were determined by HPLC to be *para*-hydroxybenzoic acid (PHBA), aniline and phenol. Furthermore, the following compounds were present at a lower concentration in the day one sample compared to the day four and day six samples: diphenylguanidine (DPG), 2-hydroxybenzothiazole (BTOH), benzothiazole (BTH), 2-mercaptobenzothiazole (MBT) and 2-methylmercaptobenzothiazole (MeMBT).

No historical data existed for PHBA, so a three-month study was undertaken to determine the normal level of PHBA in the feed to the main WWTP. The findings were that PHBA levels varied over a wide range and on irregular occasions was found to be totally absent from the feed. Other components also varied but generally were always present. Day one would appear to have been one of those rare occasions when PHBA was absent from the feed and this coincided with the absence of two other components, aniline and phenol.

The analysis (using HPLC method 2) of the day one and day one Vitox feed samplings are shown in Figures 6.4 and 6.5 (overleaf). The chromatograms have been overlaid for ease of comparison between the various samples.

HPLC analytical conditions for the above analysis were based on the same eluent, gradient programme and column. However, a different HPLC pump module had to be used due to failure of the Varian Vista 5500. The new model, a Varian Star LC pump, had subtly different pumping characteristics and dwell volume which produced a chromatogram with slightly shifted retention times for the various components.

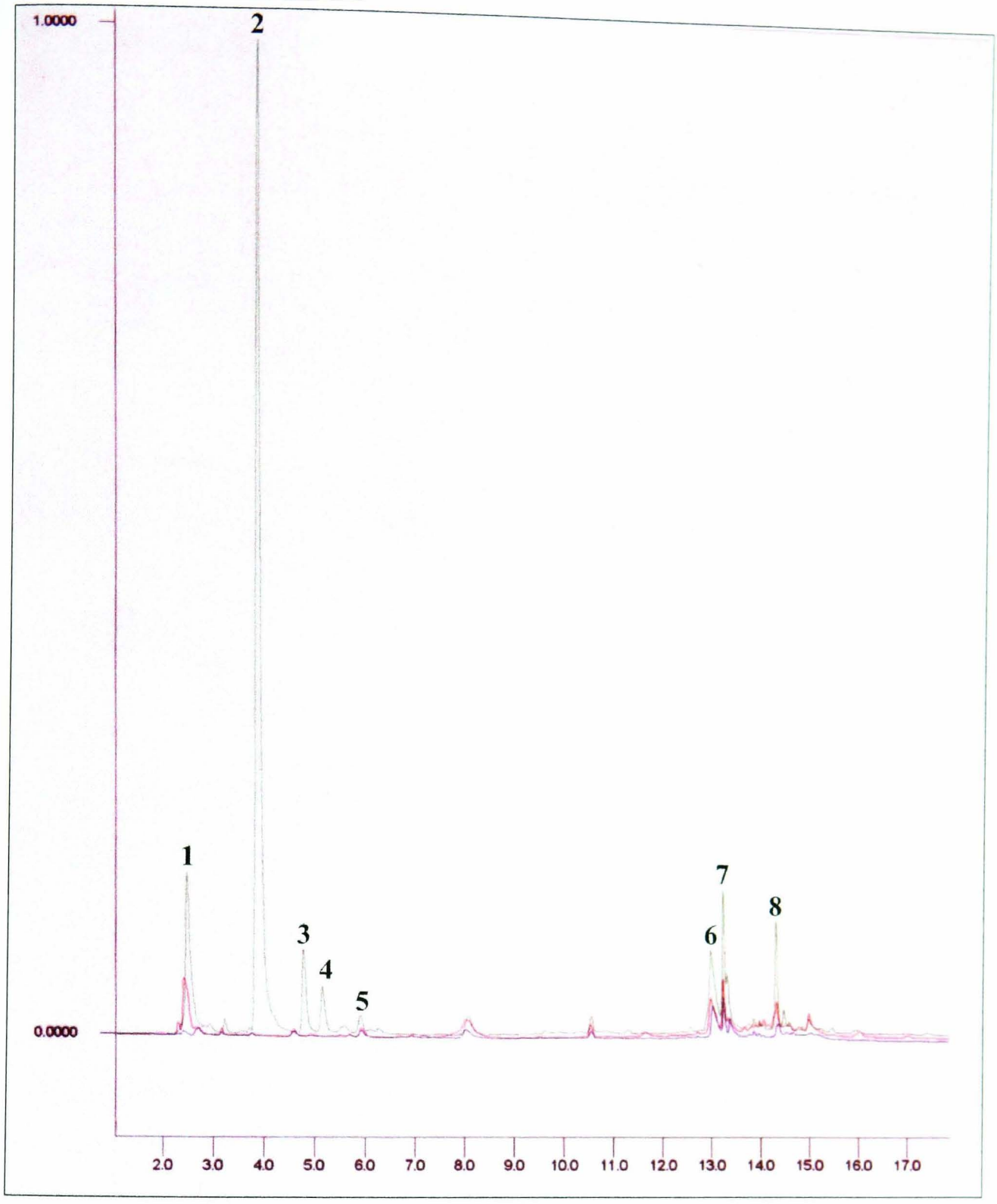


Figure 6.4 Laboratory biological reactor Vitox feeds: reactor R4 (PEG reactor) day one pm, (blue trace); reactors R1 and R2, day one am (red trace); reactors R1 and R2, day four am (green trace). Key: **1** diphenylguanidine (DPG); **2** *p*-hydroxybenzoic acid (PHBA); **3** aniline; **4** phenol; **5** 2-hydroxybenzothiazole (BTOH); **6** benzothiazole (BTH); **7** 2-mercaptobenzothiazole (MBT); **8** 2-methylmercaptobenzothiazole (MeMBT).

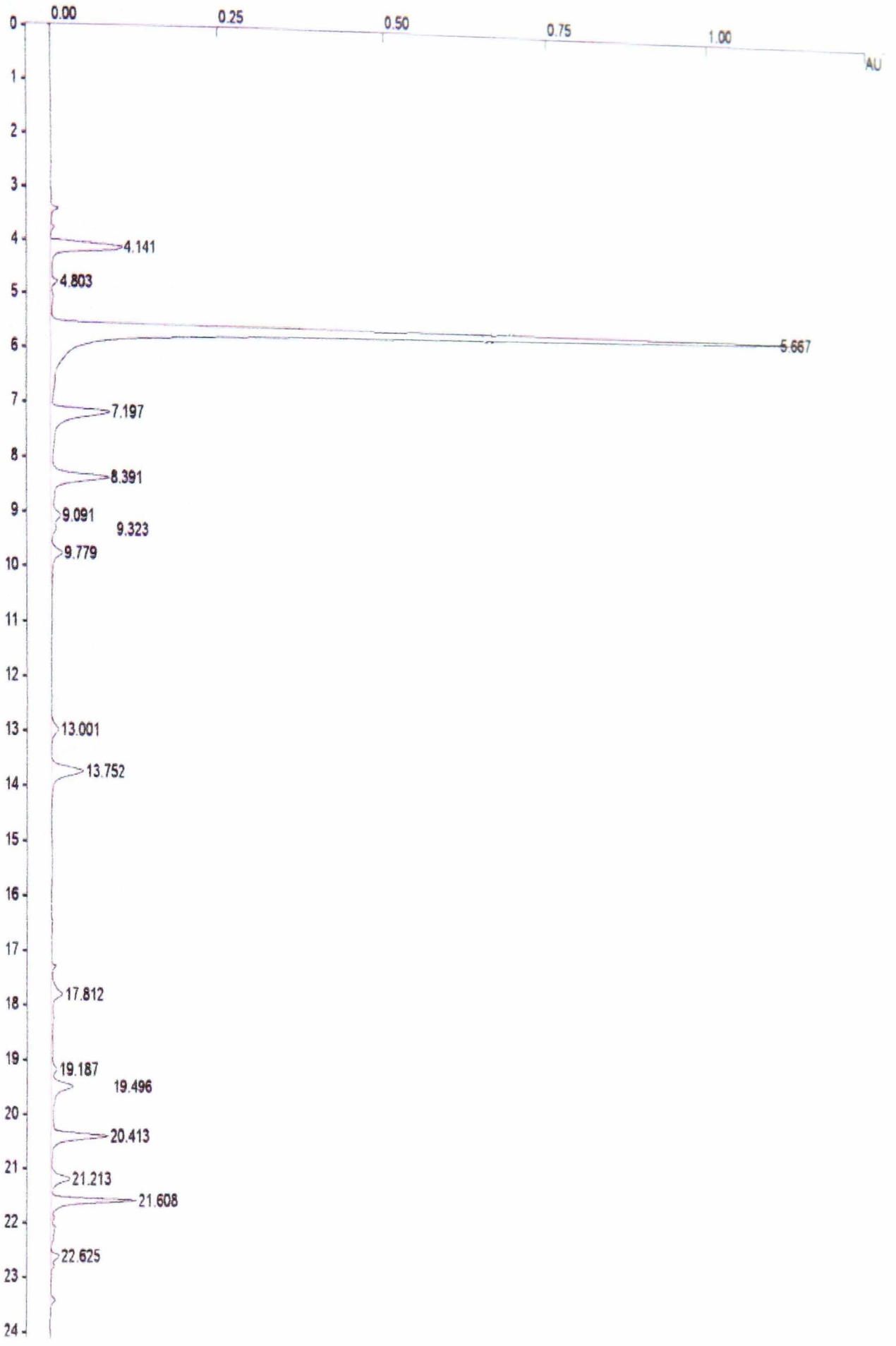


Figure 6.5 HPLC chromatogram for typical 'Vitox' feed to main WWTP. See Table 6.4 for peak identification and quantification.

Table 6.4 Peak identities for components in chromatogram of typical Vitox feed to main WWTP.

Peak retention time/mins	Peak identity	Component amount mg/l +/– 0.1 mg/l
4.141	diphenylguanidine (DPG)	15.0
5.667	4-hydroxybenzoic acid (PHBA)	282.0
7.197	aniline	11.3
8.391	phenol	13.9
9.779	2-hydroxybenzothiazole (BTOH)	7.4
13.001	benzothiazole (BTH)	3.3
13.750	2-mercaptobenzothiazole (MBT)	32.7
17.812	diphenylurea (DPU)	1.8
20.413	benzothiazole-2-sulphenic acid	29.1
21.608	benzothiazole-2-sulphonic acid	30.2

6.9 Laboratory induced colour production in bioreactors

Analysis of the events leading up to and during the accidental colour production by the laboratory bioreactors indicated that there may be more than one mechanistic route to the red chromophore. Historically the red colour has been associated with benzothiazoles and in particular 2-mercaptobenzothiazole (MBT). De Wever (1995) demonstrated that MBT and the methyl analogue (MeMBT) give rise to a red coloured effluent from an activated sludge treatment system.

To test the hypothesis of a second mechanistic route to the red chromophore, it was decided to prepare a synthetic feed that would be lacking the components as determined in the HPLC analysis of Vitox feed on day one which predated the colour formation.

A synthetic feed was based on the flow ratios for the various manufacturing plants on site was prepared. Samples of the various effluent streams were obtained from each manufacturing unit and blended to produce a feed that matched normal Vitox feed (Table 6.5). Also a feed was prepared that matched that fed to the laboratory reactors on day one as sampled from the WWTP control room. This feed was lacking components from the PHBA and Flectol TMQ manufacturing plants (see Table 6.6 for composition of this feed).

Table 6.5 Synthetic feed based on plant effluent ratios (normal Vitox feed).

Plant	Effluent flow m^3/day	Ratio
Flectol TMQ	45	0.14
MBS	144	0.44
PHBA	100	0.30
DPG powder	43.2	0.131
DPG granules	144	0.44
DPG CNCl	45	0.14
PVI	100	0.30
MBTS	330	1.00
MBT	40	0.12
TCC	27	0.10

*Volume required to run for 4 days @ 3 litres/day consumption is 12 litres in total.

A three litre volume of the synthetic mix was required to produce a sufficient volume, after dilution, for the experiment. The dilution ratio for the stock mixture was based on main WWTP dilution rates for strong liquor i.e., 1:4. The strong liquor was diluted 1:4 with weak incoming liquor prior to being fed onto the biological reactors. The composition of the mixture used in the experiment based on the above table of plant effluent volumes is given in Table 6.6.

Table 6.6 Synthetic feed to match the WWTP feed as sampled on day one.

MBS	550 cm^3
DPG powder	164 cm^3
DPG granules	550 cm^3
DPG CNCl	175 cm^3
PVI	375 cm^3
MBTS	1250 cm^3
MBT	150 cm^3
Total volume	3214 cm^3

*Dilution with 12 856 cm^3 water to produce a total working solution volume of 16 072 cm^3.

The feed mixture in Table 6.6 above was fed to bioreactor R2 for three days to simulate what happened in the laboratory the first time that red colour was induced in the system. On the fourth day the feed was switched back to a 'normal Vitox' feed (Table 6.4).

Containing all of the components that are usually present, the feed was first checked by HPLC to confirm the composition was normal, in that it contained the usual levels of components such as PHBA, MBT etc. The bioreactor R1 was fed normal Vitox feed during the whole of the experiment as a control unit.

As predicted, the colour in the reactor R2 settler turned red 24 hours following the return to normal Vitox feed; the colour continued to develop for the rest of the day and as before also faded within 24 hours after initial onset of colour production.

The success of the experiment confirmed the original hypothesis that there is more than one mechanistic route to the red chromophore. However, difficulties in confirming the identity of this compound mean it could be that two different compounds are responsible for the red colour, one based structurally on MBT and the other based on PHBA.

6.10 Fill and draw experiments on colour production

In showing that colour production can be induced by PHBA, a series of simple experiments were run to evaluate other substrates as possible precursors to colour production by the biomass.

A series of two litre conical flasks were filled with one litre of activated sludge from the main WWTP. Each was supplied with nutrient and an adequate supply of oxygen. To each flask was added 50 mg of the following: MBT, BTH, phenol, Flectol TMQ, DPG and PVI. A control flask was set up to which no other substrates were added other than nutrient and an oxygen supply. Table 6.6 gives the results of the colour analysis and Figure 6.6 shows the development of colour with time.

Table 6.6 Colour measurements in Absorbance Units (AU) at 510 nm.

Time hours	Control	MBT	BTH	Phenol	Flectol TMQ	DPG	PVI
0	0.285	0.285	0.290	0.283	0.286	0.281	0.286
8	0.290	0.850	0.550	0.430	0.510	0.320	0.340
16	0.295	1.050	0.760	0.560	0.630	0.360	0.420
24	0.302	1.160	0.920	0.630	0.720	0.410	0.510
36	0.308	1.280	0.980	0.710	0.850	0.520	0.620
48	0.318	1.420	0.830	0.820	0.930	0.480	0.740
72	0.323	1.680	0.740	0.830	1.010	0.410	0.870

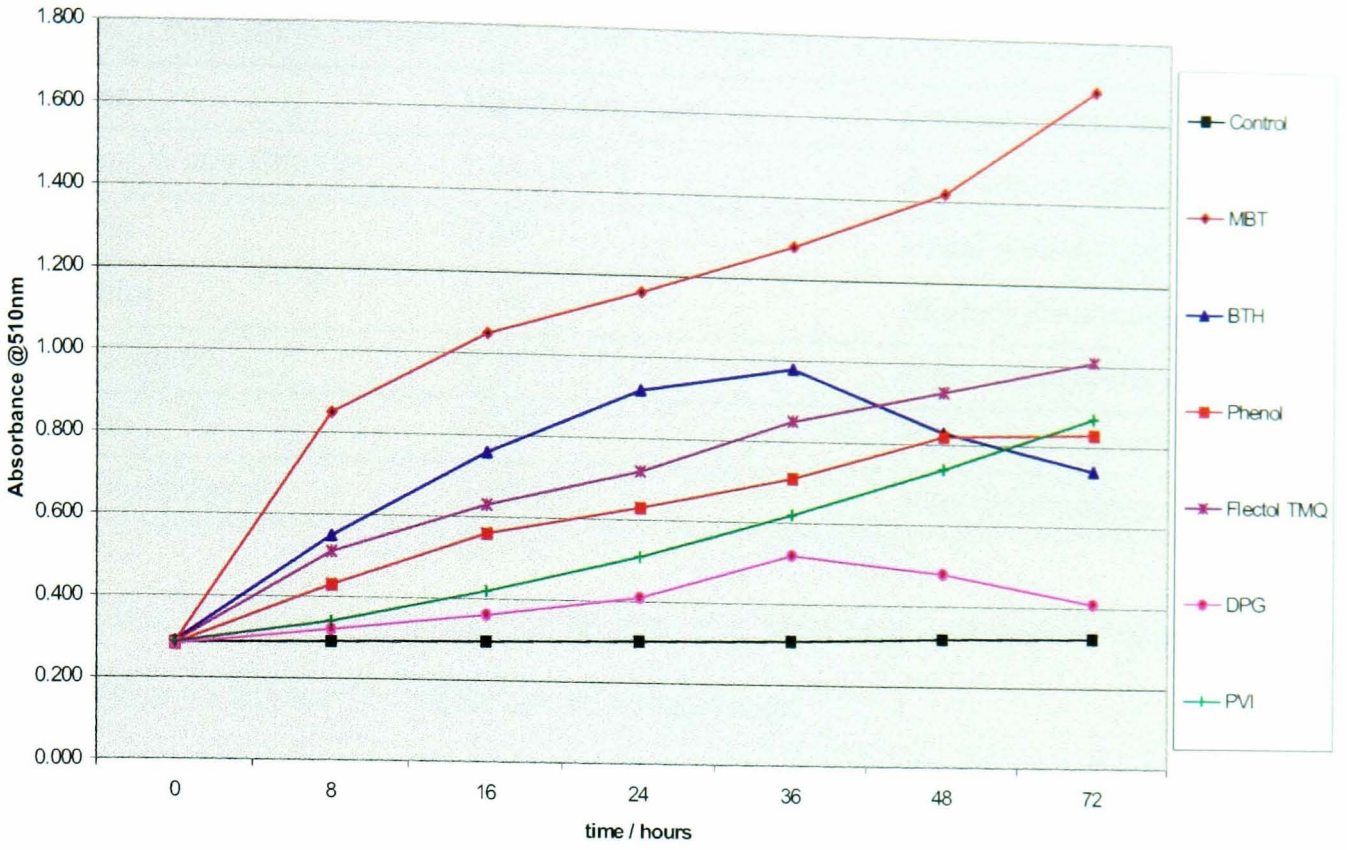


Figure 6.6 Development of colour with time.

The results support the hypothesis that colour is not due just to MBT interactions with the biomass. However, it is obvious that MBT is important in the colour production process in that the presence of MBT gave rise to the most intense colour production.

Structurally the above compounds are all substituted aromatic molecules and so it is feasible that some commonality may exist along the biological pathway leading to a common fragment. From this common fragment it is possible to postulate that a common enzyme substrate complex (ES) is formed. This in turn generates the end product, which is associated with the 'red' colour observed in the effluent from the bioreactors.

6.11 Conclusions

The findings of the various analyses are summarised in Table 6.7. It is clear from the table that the coloured components present in the effluent posses the following characteristics.

- Both chromophores appear be polar in nature;
- Both carry a negative charge and the yellow coloured fraction may posses a weaker charge compared to the red coloured component.

Table 6.7 Analytical summary for colour investigation: solvent extraction studies.

Solvent	Dipole constant	Colour extraction
Acetone:Water (90:10)	1.84 (H_2O)	Best colour extraction
Acetone	2.88	Good colour extraction
Methanol	1.70	Slight colour extraction
Ethyl acetate	1.78	Very little colour extracted
Toluene	0.375	Very little colour extracted
Dichloromethane	1.074	Very little colour extracted

The results of solid phase extraction (Figure 6.8) need to be considered with reference to the sorption mechanism for each type of sorbent used.

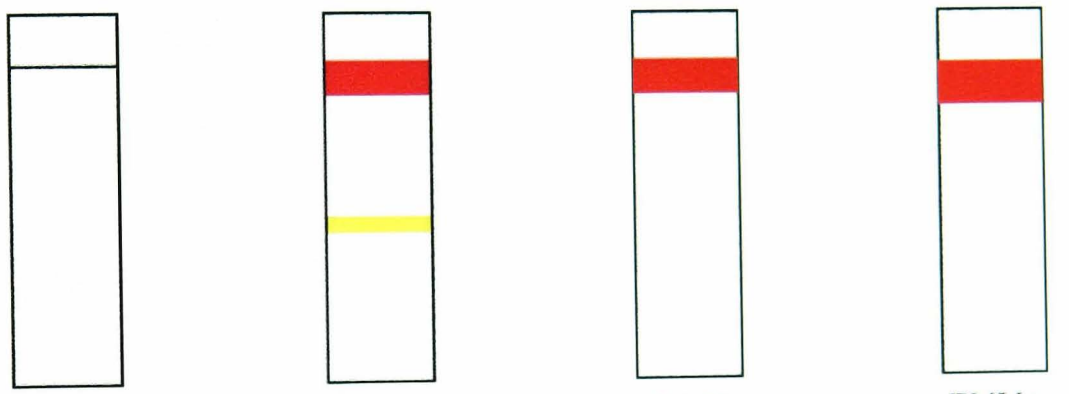
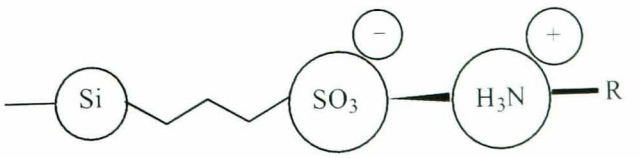


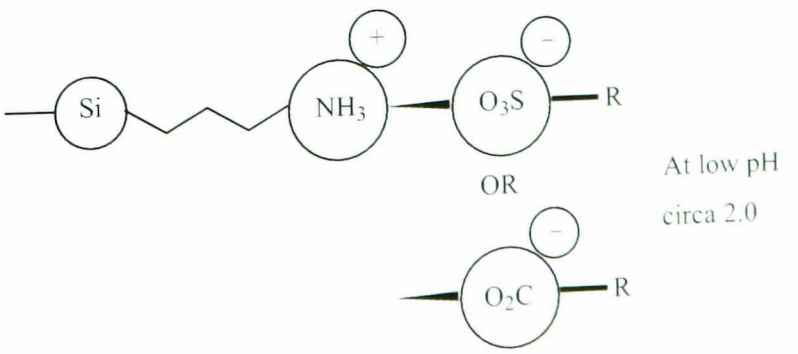
Figure 6.8 Solid phase extraction of colour.

However, only two of the four sorbents used need to be considered here, i.e., the PRS sorbent (a cation exchanger) and the NH_2 sorbent (a weak anion exchanger).

PRS ~ a cation exchanger



NH_2 ~ a weak anion exchanger



From these two results the following can be deduced about the nature of the chromophores present:

PRS ~ no retention of colour, therefore the chromophores do not posses any cationic charge;

NH_2 ~ good colour retention (two coloured bands), this indicates that the components retained are ionised at low pH and are anionic in character. Furthermore, one of the two chromophores carries a stronger ionic charge over the other as indicated by the yellow coloured component being weakly retained on the sorbent.

Possible candidate functional groups giving rise to the observed interactions are:

Red ~ $—SO_3^-$ this is on the basis of the strong retention at the top of the sorbent;

Yellow ~ $—CO_2^-$ the carboxylate ion would be expected to have a weaker interaction with the sorbent and therefore is only weakly retained and travels part way down the sorbent bed.

These findings are supported by the thin layer chromatographic (TLC) analysis (Figure 6.9) in that a yellow spot was observed at the solvent front when developing the TLC plate with dichloromethane and ethyl acetate.

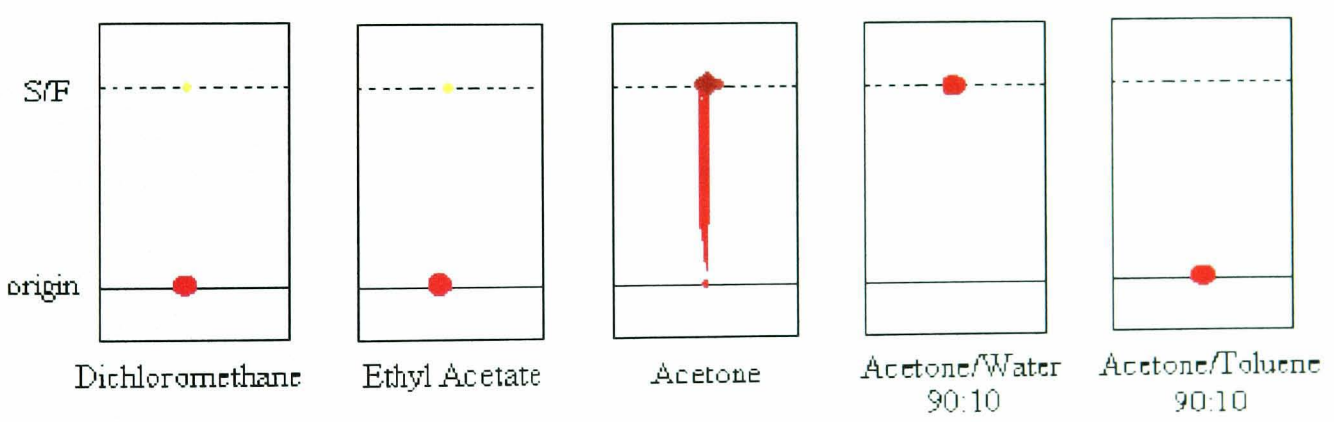
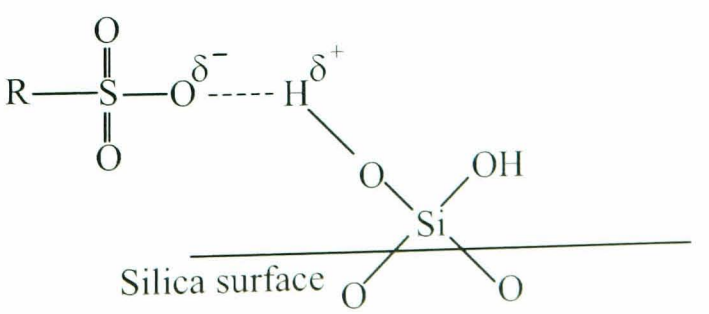
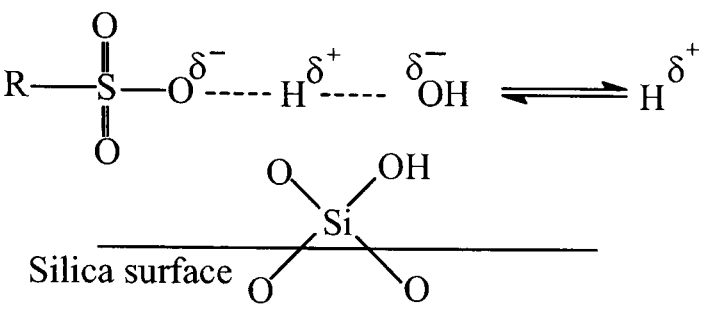


Figure 6.9 TLC studies of red coloured effluent.

A possible explanation for the presence of the brown spot with a trailing red streak from origin to the solvent front is that the red component somehow interacts with the Si-O of the TLC plate when a non-aqueous separation mode is employed, as follows.



When there are no free hydroxide ions present, as in the case of the separation using acetone (a non-aqueous) mode of separation, the chromophore has an affinity for the silica. When the same separation solvent is modified by the addition of water to produce an aqueous environment, the ionisation of water gives rise to free H^- and OH^- ions, altering the affinity for the silica.



In the first separation, in which no water is present, charge interactions between the active groups of the sample and the silanol groups of the silica sorbent are present. However, when water is added as a phase modifier the charge interactions between the silanol groups of the silica are blocked and the 'red' component migrates with the solvent front, resulting in no 'red' streak.

On the basis of the analysis carried out above it can be postulated that the substance containing the red chromophore is a polar compound which may have an ionisable functional group attached to the molecule. This functional substituent may be either SO_3^- or CO_2^-, but is more likely to be the SO_3^- on the basis of the interaction with the weak anion exchanger. The substance containing the yellow chromophore would appear to be the more common species generated by the biomass since the colour of the settler is more often than not a pale yellow to amber in colour. This component is also polar in nature and ionisable at low pH. It is only weakly retained on the NH_2 SPE cartridge, which would suggest that the charge on the molecule is not as strong as that associated with the red chromophore. This compound may possibly have an ionisable carboxylate (CO_2^-) substituent.

6.11.1 Mechanism of colour formation

To date no definitive mechanism has been proposed for the production of the red colour in the effluent from the Flexsys Ruabon wastewater treatment plant. De Wever (1995), during

treatment of mercaptobenzothiazole, observed a similar red colour in the effluent from an activated sludge bioreactor, but no attempt was made to determine either the identity of the chromophore or the mechanism of formation.

Historically it has been proposed that the red colour is a product of high MBT levels in the feed to the bioreactors. This has been based on analysis of the feed for MBT, the data collected over many years showing a tenuous link to the rise and fall in the concentration of MBT with the production of colour in the effluent. However, there are too many instances where the data (MBT concentration) is mismatched with colour *i.e.* low MBT levels with high red colour and on a few occasions high MBT levels followed by little or no red colour production.

From this it would be prudent to consider that more than one mechanism exists for the generation of red coloured effluent. However, identification of the various enzymes responsible for the biological transformations that occur within the biomass is beyond the scope of this study.

6.11.2 An alternative to MBT

In the present study, it has been shown that the compound *p*-hydroxybenzoic acid (PHBA) had an impact on the production of red coloured effluent. However, further work also demonstrated that almost any substrate could cause the biomass to produce a red coloured effluent. If we accept that red colour is the product of biologically mediated reactions, *i.e.* enzyme catalysed reactions, then the following can be postulated.

In 1903 Victor Henri proposed that an enzyme combines with its substrate molecule to form the ES (enzyme-substrate complex) as a necessary step in enzyme catalysis. This idea was expanded into a general theory of enzyme action particularly by Leonar Michaels and Mand Menten in 1913. They postulated that the enzyme first combines reversibly with its substrate to form an enzyme-substrate complex in a relatively fast reversible step.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$$

The ES complex then breaks down in a slower second step to yield the free enzyme and the reaction product P.

$$ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

In this model the second reaction is slower and therefore limits the rate of the overall reaction.

In the case under consideration the concentration of substrate S (MBT for example) is always present in a huge excess within the bioreactor compared to the concentration of the coloured body P. This conclusion is based on the concentration of the red colour in the sludge thickener (*ca* 0.3 mg/l) compared to the concentration of the MBT in the incoming feed (typically in the range 18–25 mg/l). If this is the case it would be expected that the effluent should be permanently red coloured. Since this is not the case, a second enzyme E_2 may exist that combines in a fast step with the product to produce another complex and thus reduces the colour following initial formation.

$$E_2 + P \rightleftharpoons EP$$

6.11.3 Colour production due to MBT concentration

Case 1: colour production due to MBT

The typical concentration of MBT in the feed to the bioreactors of the main wastewater treatment plant is in the range 18–25 mg/l. At this concentration the colour of the effluent is more often than not a yellow/amber. However, if the concentration of MBT rises significantly above this level (*ca* 30 mg/l or more), the resultant effluent is seen to turn red a few days later, between 36 and 48 hours after the initial MBT increase. It can be postulated that at these elevated concentrations, MBT is inhibiting a specific enzyme and/or pathway that is responsible for the reduction/removal of the coloured body in the effluent.

Consider two situations that occur for MBT: (a) typical concentrations of MBT <25 mg/l and (b) greater than 30mg/l MBT in the feed.

(a) typical concentrations of MBT (<25 mg/l)

In this case the normal enzymatic processes occur as follows.

$$E_{MBT} + S_{MBT} \rightleftharpoons ES_{MBT} \text{ complex} \qquad R1$$

$$ES_{MBT} \text{ complex} \rightleftharpoons P_1 + E_{MBT} \qquad R2 \ (P_1 \text{ Red})$$

The expulsion of product P_1 stimulates the biomass into generation of a secondary enzyme E_2 that is specific for P_1 a metabolite of the MBT biotransformation.

$$E_2 + P_1 \rightleftharpoons E_2P_1 \text{ complex} \qquad R3$$

$$E_2P_1 \text{ complex} \rightleftharpoons P_2 + E_2 \qquad R4 \ (P_2 \text{ colourless})$$

In the above scheme the initial ES_{MBT} formation step is faster than the subsequent dissociation step in which the product P_1 is formed with a concurrent release of the enzyme E_{MBT}. Similarly, the reaction step R3 is fast in comparison to R2 and so there is a net reduction of P_1. The product P1 in this case is the red coloured body observed in the effluent.

Once the enzyme E_2 is present within the biomass any further P_1 components are quickly complexed to produce the E_2P_1 complex, and thus prevent any red colour from being developed.

(b) MBT concentrations of greater than 30mg/l

In this situation, the following is postulated.

$$E_{MBT} + XS\ S_{MBT} \rightleftharpoons ES_{MBT} + \text{free unbound MBT} \qquad R5$$

$$ES_{MBT} \text{ complex} \rightleftharpoons E_{MBT} + P_1 + \text{free unbound MBT} \qquad R6$$

In this case the unbound MBT exerts an inhibiting effect on the biomass and prevents the production of enzyme E_2.

Under normal conditions P_1 would stimulate the biomass to generate the corresponding coenzyme E_2. However, due to the presence of free uncomplexed MBT this does not occur, which results in the build up of the product P_1 within the biomass resulting in the generation of the red coloured body.

The biomass slowly acclimates to the presence of the free MBT with an increased production of the relevant enzyme. This extra generation of the enzyme E_{MBT} is then able to begin complex formation as in R1. Once the formation of ES_{MBT} takes place the concentration of free MBT within the biomass is quickly reduced to a point where it no longer exerts any toxic inhibition on the biomass. At this point the biomass is stimulated to produce the coenzyme E_2 as in R2.

The compound MBT is a known biological inhibitor (De Wever *et al.*, 1994) and this would support the above postulation for colour formation due to the presence of MBT at concentrations above the norm (*ca* 18–25 mg/l).

Case 2: colour production due to p-*hydroxybenzoic acid*

The biomass at Flexsys Ruabon has a long history of treating wastewaters containing high concentrations of *p*-hydroxybenzoic acid (PHBA), typically in the range of 180–250 mg/l. The compound PHBA has long been considered a 'soft' compound in terms of biological breakdown since this compound is very quickly metabolised within the biomass. Analysis of both biomass and resulting effluent confirm this in that no or very little PHBA is present after a few hours of the initial inoculation of the biomass with PHBA, producing a series of metabolites including phenol and methyl parabens. The compound PHBA appears to have no toxicological effects on the biomass even at high concentrations, and on this basis it may be argued that any colour production occurring is not due to toxic inhibition of the biomass.

It has been shown through analysis of the incoming feed to the bioreactors that the concentration of PHBA varies over a wide range and on occasions is completely absent from the feed. This absence has been observed to persist for up to two days.

As described in section 6.8.1, this absence on one occasion was subsequently followed by the appearance of the red colour in a laboratory bioreactor analogous to the main wastewater treatment plant. It was subsequently confirmed that an absence of PHBA followed by a period in which PHBA is present gives rise to the production of a red coloured effluent.

It is assumed that a similar series of enzymatic reactions occur to those proposed above in which specific enzymes are generated within the biomass to facilitate the biotranformation of PHBA and associated metabolites. However, in the case where no PHBA is present then it may be postulated that the various enzymes and associated bacterial genera are progressively washed out of the biomass. Thus when PHBA is once again present in the feed to this biomass which has lost a significant portion of specific genera required for the biotransformation of PHBA, PHBA is observed to accumulate within the biomass and to be detected in the effluent from the bioreactor.

There is an initial lag phase in which growth of the relevant organisms takes place.

$$E_{PHBA} + S_{PHBA} \rightleftharpoons ES_{PHBA}\ \text{complex} \qquad \text{R7}$$

The reaction step R7 is analogous to R1 in the MBT reaction.

Also, as with MBT, there is now a large excess of PHBA within the biomass due to the slow acclimation of the biomass to the presence of PHBA. This excess PHBA interferes with the following reaction steps, namely R5 and R6. This is not thought to occur via toxic inhibition as in the case of MBT but rather as a consequence of 'competitive inhibition'.

As stated above PHBA is normally present at quite high concentrations (*ca* 180–250 mg/l) and at these concentrations does not inhibit biological activity. Thus it can be postulated that following an extended period of PHBA absence in the feed to the bioreactors, resulting in washout of specific bacterial genera associated with PHBA metabolisation, the following may occur.

No S_{PHBA} therefore no production of the ES_{PHBA} complex, resulting in no expulsion of the product P_1; this in turn limits the production of enzyme E_2.

When PHBA is now present the following may occur within the biomass.

$$S_{PHBA} + \text{very little or no } E_{PHBA} \rightleftharpoons \text{trace of } ES_{PHBA} + \text{large excess free PHBA} \qquad R8$$

$$ES_{PHBA} \text{ complex} \rightleftharpoons E_{PHBA} + P_1 + \text{excess free PHBA} \qquad R9$$

Enzyme E_2 now stimulated by the expulsion of trace levels of the product P_1 while in the presence of a greater excess of PHBA.

The enzyme E_2 has an affinity for both P_1 and PHBA. The PHBA is present at sufficient concentration that it binds reversibly with enzyme E_2 initially in preference to P_1. This results in the product P_1 accumulating within the biomass.

$$E_2P_1 \text{ complex} \rightleftharpoons P_1 + E_2 + S_{PHBA} \rightleftharpoons E_2S_{PHBA} \text{ complex}$$

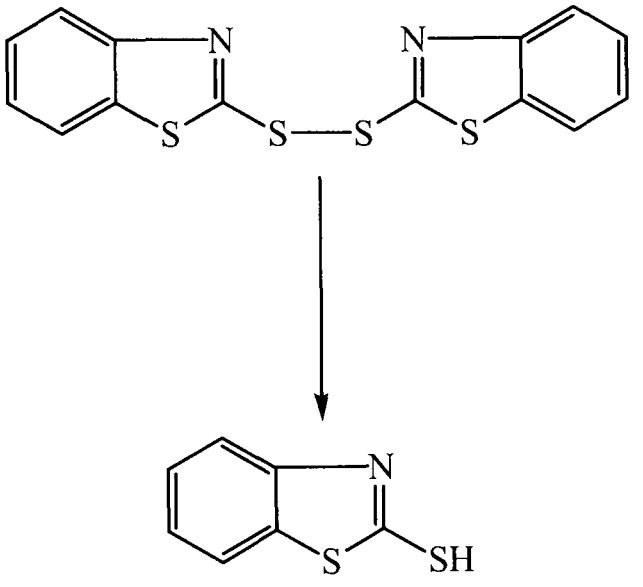
The above is an example of 'reversible competitive inhibition' and not toxicological inhibition of biological activity as is the case for MBT.

The results of both TLC and SPE analysis confirm the presence of two different chromophores. It is postulated that one is based on a sulphur centre and possibly possessing an active functional substituent group such as $-SO_3H$ and the other is based on an alternative functional grouping such as $-CO_2H$. The MBT and other benzothiazoles may be the precursor for the sulphur-centred molecule with PHBA and other organic compounds present giving rise to the carboxylic acid form. In all cases the enzymes responsible for the biotransformations are specific in each case, but show some affinity for other substrates, hence the production of red colour during the PHBA experiment. This may explain why colour is observed even when low concentrations of MBT precede a period of high colour and *vice versa*.

In the above worked examples the enzymes E_2 are not the same, the terms are used to simply differentiate between the first enzyme in R1 and that in R2. It may well be that they do have similar characteristics, but it is beyond the scope of this research to identify the various enzymes operating within the biomass.

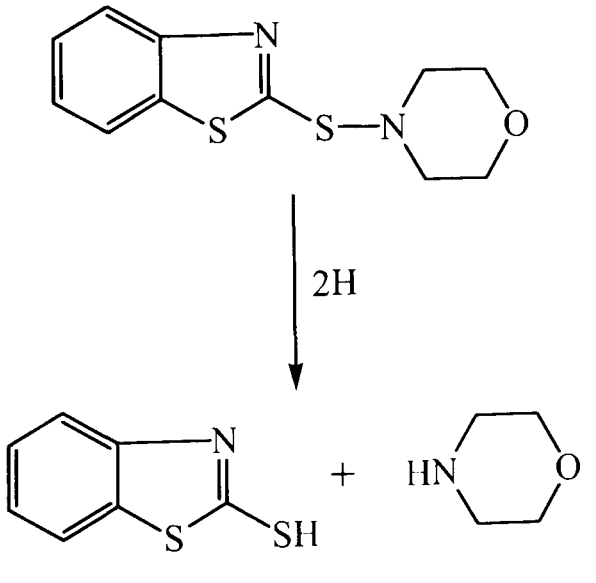
The results of the work in Section 6.10 would suggest that almost any substrate can give rise to colour within the biomass. Some of the substrates studied are known to be relatively 'soft' in terms of biological treatment and as such are assumed to be non-toxic towards the biomass at the concentrations being fed to the biomass. The colour production in these cases may be due in part to similar processes to that postulated for PHBA. It is the lack of historical analytical data on the concentrations in the feed to the main wastewater plant that prevents extension of the above theory to these compounds.

A further source of the data mismatch is the MBT analysis of the influent to the bioreactors. The analysis of the feed to the biomass determines only that MBT which exists as free MBT and does not account for MBT available from other sources. Other sources of MBT include the reductive breakdown of any MBTS present in the biomass.



Reduction of the S-S bond yielding 2 moles of MBT

MBT is also liberated during the biodegradation of morpholinobenzothiazole.



A further source of MBT is through the metabolisation of BTOH in the presence of BTH. In this pathway MeBTH is a precursor to the production of a mixture of MBT, BTH and MeBTH in the ratio 0.5:0.3:0.2 respectively.

It is probable that these extra sources of MBT give rise to the unexpected colour production that is observed when the MBT levels in the feed are apparently low.

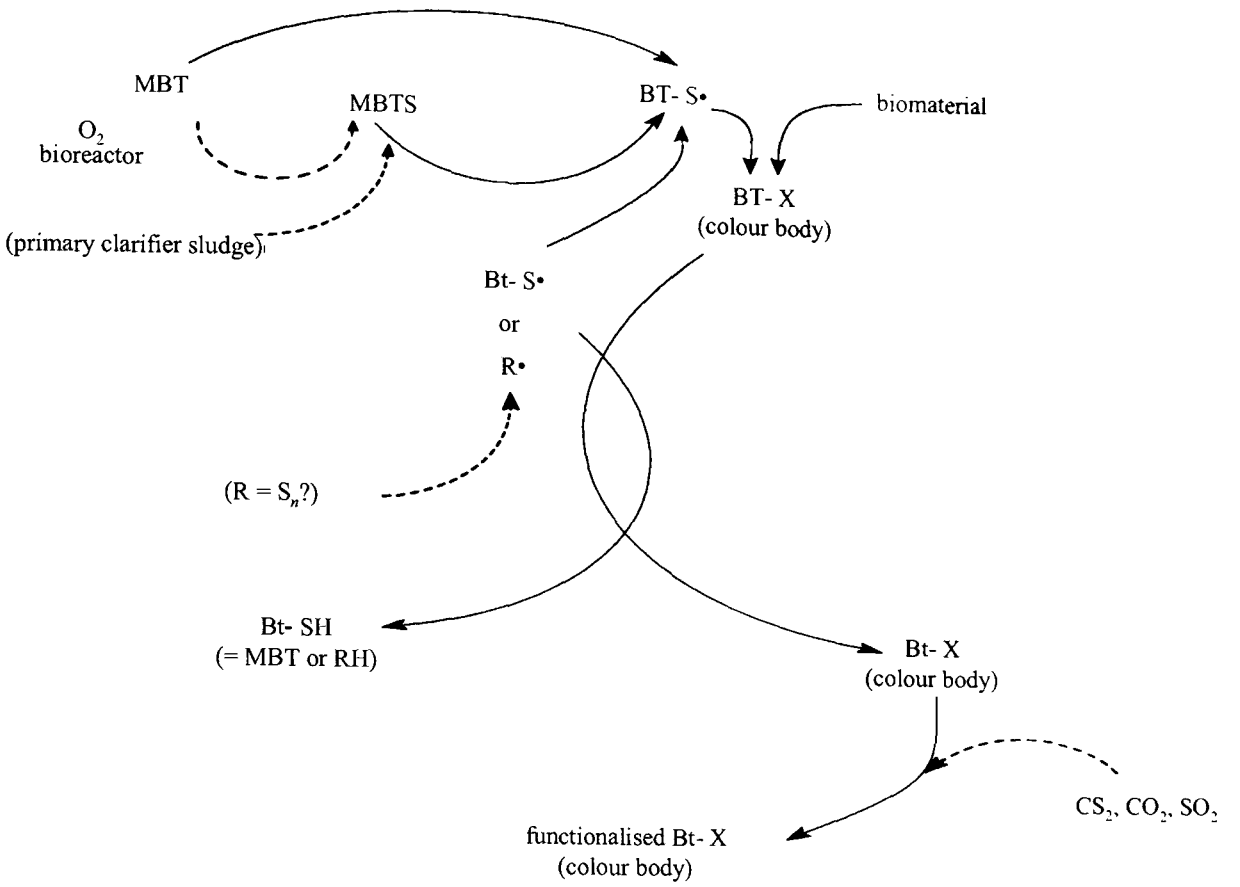
6.11.4 Investigation of the colour phenomenon at Deventer Research Laboratories

During 1999 the Flexsys Research Laboratories at Deventer in The Netherlands were contracted to carry out an investigation into the possible mechanism for formation and identity of the red chromophore. Work at the Ruabon laboratories could not proceed any further due to lack of necessary analytical facilities.

Extensive analytical investigations at Flexsys Deventer using such techniques as nuclear magnetic resonance (NMR) spectroscopy and flow-injection mass spectrometry generated much new knowledge about the possible mechanism for colour formation within the biomass (Nijhuis and Maslow, 1999). From their results, the investigators drew the following conclusions.

- MBT radicals (or other sulphur-centred radicals) may be formed from MBT or by homolytic scission of MBTS (or other disulphide-containing compounds).
- Besides sulphur-centred MBT radicals, other MBT radicals can be formed with the functional group on the aromatic ring in the 5-position (•BT-SH), such as $-CO_2H$ or $-SO_3H$.
- Water soluble colour bodies are formed by reaction of MBT radicals with biomaterial.

The following schematic gives an overall representation of a possible mechanism proposed by Dr Walter Nijhuis (Nijhuis and Maslow, 1999):



The findings of the research group at Deventer (Nijhuis and Maslow, 1999) are supportive of some of the findings of this study in that they too found that the 'red' coloured compound is charged and that this charge is likely to be due to the presence of either $-SO_3^-$ and/or $-CO_2^-$ functionality. They also found that the coloured product can be isolated using a charged sorbent similar to the IST NH_2 SPE cartridge. NMR studies indicated that the coloured substance might be structurally related to MBT and BTH with both containing a substituent such as SO_3H or CO_2H on the ring in the 5 position. They also confirmed the fact that the colour tended to be formed under anaerobic conditions and highly oxygenated environments tended to limit colour formation.

The research at the Flexsys Deventer laboratories also indicated that a protein fragment formed part of the molecular structure of the coloured product. This would explain the difficulty experienced in trying to isolate the coloured fraction, since the laboratories at Flexsys Ruabon do not have the facilities to perform protein sequencing.

References for Chapter 6

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CHAPTER 7
CONCLUSIONS

7.1 Biodegradation of benzothiazoles

Research into the biodegradation of a wide variety of xenobiotic compounds has been carried out by a large number of groups. However, focusing on benzothiazoles in particular, while there is an extensive literature on their production and use, there have been relatively few reports before this of their biodegradation. Notable among these are the following.

Biodegradation studies on benzothiazole-2-sulphonic acid (BTSA) were carried out by Mainprize *et al.* (1976) and on 2-hydroxybenzothiazole by De Vos *et al.* (1993a). 2-Mercaptobenzothiazole (MBT) was studied by Chudoba *et al.* (1977) and by De Wever and Verachtert (1994). Chudoba *et al.* suggested that MBT may be recalcitrant to biodegradation, while Mainprize *et al.* (1976) and Gaja (1996) proposed that it may be partially metabolised by using a biomass that has been acclimated to other benzothiazoles as a carbon source.

However, all these studies used batch reactors. They are therefore inadequate as models for full-size activated sludge wastewater treatment plants, which are continuous flow systems. The three model reactors used in this study were designed to be continuous flow systems, thereby modelling more closely the full-size plant. In addition, the biomass used in the model reactors was obtained from an operating wastewater treatment plant which further means that the results obtained should be a better guide to those that occur in the full-size system.

Mixed culture bacteria were taken from the plant reactors and acclimated while being fed with 'normal' feed taken from the main wastewater treatment plant. The data obtained demonstrated that the three model reactors operated almost identically and produced values of a similar magnitude to those of the main water treatment plant, thereby confirming that they constituted good models for the full-size plant.

A synthetic feed was then prepared, the composition of which was based on that of the normal feed going onto the main biological reactors, but without the 'rubber chemicals'. The prepared biomass in reactor R3 was then fed this synthetic feed for a period of six months. This was to ensure complete removal of any rubber chemicals and associated metabolites. After two months of being fed this synthetic feed the biomass lost its characteristic brown/purple appearance and became very light coloured (a light sandy colour). The final colour of the biomass was almost white and the floc had a light fluffy appearance with excellent settling properties. The colour of effluent from this biological reactor was water-white, in contrast to that from reactors R1 and R2, which varied from pale straw to a deep amber colour.

Analysis of effluent from reactor R3 confirmed that the washout of all traces of rubber chemicals and associated metabolites had been successful. In all samples examined during the last three months of acclimation to the synthetic feed, no traces of rubber chemicals or metabolites were detected. The biomass thus prepared was then used for specific metabolic pathway studies on how various substrates and substrate mixtures are broken down. This was done by feeding the biomass of reactor R3 with the base matrix plus specific substrates added at various concentrations, typically 25, 50 and 100 mg/l.

A wide range of benzothiazole compounds were studied singly and in various combinations (in pairs, in mixtures containing three components and one mixture of four substances). In addition the biological degradation of a selection of five non-thiazolic compounds was also investigated. The results of these studies allowed the following general conclusions to be drawn.

- As well as confirming some of the previous studies, possible novel metabolic pathways were identified. What is clearly apparent from the results of all the studies is that biological activity, and in particular specific enzymatic pathways, are affected not only by the incoming substrate but also by the generated metabolites.

- Metabolisation of mixtures showed interdependence between substrates with initial mutual inhibition (though occasionally mutual stimulation of degradation occurred).

However, acclimation usually meant degradation eventually took place. Different results were found for the model flow reactors used in this study compared with the results obtained previously from batch studies.

- The biomass acclimated to the composition of the feed. It seems that the biomass obtained from the plant retained a 'memory' of its history, even when fed the base matrix, such that when subsequently fed substrates that are components of the main plant waste stream, the biomass developed the ability to degrade even so-called 'recalcitrant' compounds.

- Similarly, though initially being inhibited by extremes of pH, the biomass appeared to develop an ability to continue with the process of nitrification at lowered pH following a period of low pH. This could have been due to acclimation or simply the effect of other organisms carrying out the process of nitrification. The evidence favoured the explanation that continuing nitrification is due to other organisms present that are capable of nitrifying at the lower pH. A second possibility also exists, namely that the low pH prevented the degradation of nitrogenous material to produce ammonia within the biological reactor.

- Contrary to the findings of some previous researchers, it was found that the biomass inoculated with the various benzothiazoles continued to fully nitrify in all cases.

- Previous studies on inhibition of the nitrification process have been done in the presence of inhibitors at a given concentration within the biological reactor. This study looked at how the inhibitory compound behaved in a system which was efficiently degrading all of the thiazoles so their concentration in the biological reactor was effectively zero and hence they had no inhibitory effect – irrespective of their concentration in the feed.

- This appears to be the first report of aerobic degradation by a mixed culture activated sludge of 1,2-dihydro-2,2,4-trimethylquinoline (Flectol A). This study demonstrated that the monomer is biodegradable, albeit after a suitable acclimation period, with the production of biodegradable metabolites.

- This also seems to be the first systematic study of the biodegradability of diphenylguanidine (DPG). Following a prolonged absence of DPG from the feed to the biomass, as in the case of reactor R3, the necessary enzymes for DPG metabolisation were lost from the biomass. Following reintroduction of DPG to the biomass significant levels of DPG were detected in the effluent confirming that the enzymes necessary for DPG removal were lost over time. However, once the required enzymes had been stimulated, the DPG was quickly metabolised as indicated by the production of a series of metabolites.

- Despite exhaustive literature searches no published data could be found concerning industrial aerobic degradation of PVI using the activated sludge process. The situation was no different regarding the various metabolites generated during the study. The results of this study have therefore generated a unique data set relating to the specific metabolisation of PVI and associated metabolites by an aerobic biomass.

- The product PHBA has a long history of manufacture at the Flexsys Rubber Chemicals site in North Wales. However, as it has traditionally been thought to be readily biodegradable, no previous studies had been carried out into its metabolisation. Analysis of the effluent discharges from the PHBA manufacturing plant revealed that concentrations of PHBA exceeded 3000 mg/l. However, analysis of effluent from the wastewater treatment plant showed PHBA concentrations below the current limit of detection (0.1 mg/l) confirming that it was indeed readily biodegradable.

- Similar findings have been reported by other workers in the field studying the biotransformation of both PHBA and associated esters. Valkova *et al.* (2001) reported that PHBA was biotransformed into phenol via a decarboxylation step. According to Valkova *et al.* this decarboxylation of *p*-hydroxybenzoic acid into phenol by aerobic bacteria had previously only been reported once using a strain of *E. aerogenes* (Steigerwatt *et al.*, 1976) under both aerobic (Patel and Grant, 1969) and anaerobic conditions (Grant and Patel, 1969).

7.2 The red colour phenomenon

The specific biomass at the Flexsys Ruabon plant produced on many occasions a highly coloured effluent, which in the past led to the serving of a writ from the Riparian owners of the River Dee adjacent to the site. It was originally thought that the colour problem was unique to the Ruabon site. However, it subsequently came to light that similar treatment plants within the Flexsys organisation also produced a red coloured effluent.

De Wever (1995) and De Vos (1993b) also reported the formation of a red coloured effluent from a model system using an 'activated sludge' to treat benzothiazole-containing wastewaters.

This colour formation was a purely aesthetic problem but still problematic for the management at the Flexsys facility. It had been suggested that MBT was the principal component responsible for the formation of the 'red' chromophore. Neither De Vos nor De Wever identified the chromophore responsible for the red colouration but did suggest conditions favourable to its formation. They proposed that certain combinations of benzothiazole (BTH) and 2-hydroxybenzothiazole (BTOH) gave rise to the red coloured effluent.

The conclusions of this research are that the 'red' chromophore is the product of a number of related events occurring within the biomass, namely:

(i) a sudden change in the concentration and composition of the influent matrix;

(ii) a prolonged absence of a substrate followed by its reintroduction into the feed matrix.

Furthermore, it was shown that the Ruabon biomass generates two coloured chromophores, a predominant 'yellow' component and the 'red' coloured component. It is proposed that these two coloured substances have the following characteristics:

(i) the red component contains an SO_3^- functionality associated with a protein-thiazole complex;

(ii) the yellow chromophore has a carboxylate CO_2^- functional group associated with a similar but different protein-thiazole complex.

References for Chapter 7

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